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

Christopher G. Wilson[1]*, Reuben W. Nowell[1], Timothy G. Barraclough[1]

[1] Department of Life Sciences, Imperial College London, Silwood Park Campus, Buckhurst Road, Ascot, Berkshire, SL5 7PY, UK

* Corresponding author: chris.wilson@imperial.ac.uk

Summary

Bdelloid rotifers are microscopic invertebrates thought to have evolved for millions of years without sexual reproduction. They have attracted the attention of biologists puzzled by the maintenance of sex among nearly all other eukaryotes. Bdelloid genomes have an unusually high proportion of horizontally acquired non-metazoan genes. This well-substantiated finding has invited speculation that homologous horizontal transfer between rotifers also may occur, perhaps even 'replacing' sex. A 2016 study in *Current Biology* claimed to supply evidence for this hypothesis. The authors sampled rotifers of the genus *Adineta* from natural populations and sequenced one mitochondrial and four nuclear loci. For several samples, species assignments were incongruent among loci, which the authors interpreted as evidence of "interspecific genetic exchanges". Here, we use sequencing chromatograms supplied by the authors to demonstrate that samples treated as individuals actually contained two or more divergent mitochondrial and ribosomal sequences, indicating contamination with DNA from additional animals belonging to the supposed "donor species". We also show that "exchanged" molecules share only 75% sequence homology, a degree of divergence incompatible with established mechanisms of recombination and genomic features of *Adineta*. These findings are parsimoniously explained by cross-contamination of tubes with animals or DNA from different species. Given the proportion of tubes contaminated in this way, we show by calculation that evidence for "intraspecific horizontal exchange" in the same dataset is explained by contamination with conspecific DNA. On the clear evidence of these analyses, the 2016 study provides no reliable support for the hypothesis of horizontal genetic transfer between or within these bdelloid species.

Introduction

The maintenance of sexual reproduction is a fundamental evolutionary problem (Maynard Smith 1978). Despite many costs (Lehtonen et al. 2012), sex is nearly universal among eukaryotes (Speijer et al. 2015), whereas obligately asexual lineages are rare and typically short-lived (Bell 1982; Burt 2000). Various genetic and ecological hypotheses have been proposed to explain this paradox (Kondrashov 1993; Hartfield & Keightley 2012) but definitive tests are lacking, in part because of difficulties identifying appropriate study systems (Lehtonen et al. 2012; Meirmans et al. 2012).

One approach is to investigate groups that seem to have evolved without sex over extended timescales (Judson & Normark 1996; Normark et al. 2003). Whatever mechanism typically suppresses asexuality ought to be absent or unusually mitigated in these exceptional groups, whose genetics and ecology may thus illuminate the rules that maintain sex elsewhere. However, the remarkable claim of longstanding asexuality must first be established rigorously. Only a handful of 'ancient asexual' candidates have been identified, and some are subject to ongoing doubt (Lunt 2008; Schurko et al. 2009), especially when extant populations continue to invest in males (Palmer & Norton 1991; Smith et al. 2006; Schwander et al. 2013).

Rotifers of the Class Bdelloidea have attracted particular attention in discussions of asexuality. These microscopic filter-feeding invertebrates live in nearly every freshwater habitat worldwide, however tiny or ephemeral, thanks to their tolerance for extreme conditions, including complete desiccation (Ricci 1987). The main indication of longstanding asexuality is the absence of evidence for males in nearly 500 described species (Segers 2007) despite centuries of observation (Hudson & Gosse 1886; Mark Welch et al. 2009; Birky 2010). Cytological evidence is also suggestive: *Philodina roseola* has 13 chromosomes, including three without apparent morphological homologs, and oogenesis does not seem to involve reduction or synapsis (Hsu 1956; Mark Welch & Meselson 1998).

Several unusual features of bdelloid genomes have been considered as possible consequences of longstanding asexuality. Bdelloids have far fewer vertically transmitted retrotransposons than other animals (Arkhipova & Meselson 2000; Flot et al. 2013), as predicted by selfish DNA theory when sex is absent (Hickey 1982). Deeply divergent gene copies in certain bdelloid species were initially interpreted as former alleles on previously paired chromosomes that had been evolving independently for millions of years without recombination (Birky 1996; Mark Welch & Meselson 2000; Pouchkina-Stantcheva et al. 2007). However, later work clarified that bdelloids are ancestrally

69 tetraploid, with most genes forming two divergent pairs of nearly identical sequences (Hur et al.
70 2009; Mark Welch et al. 2009). By convention, a typical gene has a closely related 'homolog', and a
71 pair of distantly related 'ohnologs' (Flot et al. 2013). Each homologous pair diverges deeply from its
72 ohnologs, and the two lineages seem too distant for genetic exchange (e.g. homology is 86% for
73 ohnologs of the heat-shock gene *hsp82* in *P. roseola*). Within pairs, homologous copies are similar or
74 identical, which implies some ongoing homogenising mechanism whose nature is as yet unclear. It
75 has been speculated that homologs serve as reciprocal templates for the repair of DNA double-
76 strand breaks (DSB) induced by desiccation, and that break-induced replication, recombination and
77 gene conversion might mediate concerted evolution between them (Gladyshev & Meselson 2008;
78 Mark Welch et al. 2008; Flot et al. 2013; Hespeels et al. 2014). These structural features and
79 hypothetical mechanisms are central to current models of bdelloid genome evolution.
80
81 Bdelloid genomes have a very high proportion of genes of non-metazoan origin, which indicates
82 exceptional rates of horizontal gene transfer (HGT) from foreign sources (Gladyshev et al. 2008).
83 Initial claims of this kind in tardigrades (Boothby et al. 2015) have been rejected as artefacts of
84 contamination after more careful analysis (Delmont & Eren 2016; Koutsovoulos et al. 2016; Richards
85 & Monier 2016). In bdelloids, however, extensive inter-kingdom HGT is consistently supported by
86 independent lines of evidence in multiple species, including datasets and analyses that exclude
87 contamination as an explanation (Gladyshev et al. 2008; Boschetti et al. 2012; Flot et al. 2013; Crisp
88 et al. 2015; Eyres et al. 2015). Most foreign genes appear bacterial in origin, but fungal, plant and
89 archaeal genes also are represented (Boschetti et al. 2012). Animal genes have probably been
90 acquired horizontally too, but these are not readily distinguished against a metazoan background.
91
92 How and why bdelloids have accumulated so many foreign genes is unclear. One hypothesis is that
93 environmental DNA may be incorporated during recovery from desiccation, if cell membranes are
94 compromised and genomes require extensive repair (Gladyshev et al. 2008; Hespeels et al. 2014).
95 However, at least one desiccation-tolerant tardigrade shows no such accumulation (Hashimoto et al.
96 2016), whereas some desiccation-intolerant bdelloids continue to acquire foreign genes (Eyres et al.
97 2015). Although the rate of stable incorporation is high relative to other animals, it is estimated to
98 be low in absolute terms: on the order of ten events per million years (Eyres et al. 2015).
99
100 When first describing non-homologous HGT into bdelloids from other Kingdoms, Gladyshev et al.
101 (2008) speculated that "there may also be homologous replacement by DNA segments released
102 from related individuals", in which case "bdelloid rotifers may experience genetic exchange

resembling that in sexual populations". This idea was extended by Flot et al. (2013), who speculated that HGT “from rotifer to rotifer” may have “replaced” functions otherwise performed by sex. Here, we discuss a study appearing in *Current Biology*, which claims to support this view by supplying evidence of extensive transfer of genetic material between individual bdelloid rotifers.

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

Most 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 authors interpret this incongruence as "strong evidence" of "multiple cases" of "interspecific horizontal genetic transfers" from "donor species" to "recipient individuals." In other samples from the same dataset, the authors report “allele sharing” within “haplotype trios” of conspecific individuals, which they interpret as evidence of “intraspecific horizontal exchange”. They conclude that they have discovered "an unexpected (and possibly unique)...ameiotic strategy of genetic exchange and recombination among asexual, morphologically female organisms", which they "propose here to call 'sapphomixis' (from the name of the Greek lesbian poetess Sappho and mixis 'mingling')."

The dataset features some surprising patterns that seem to require clarification. In every case of reported incongruence, the inferred donor species was recovered from the same maple or plane tree as the putative recipient at the time of sampling (Debortoli et al. 2016; Table S3). This is quite unexpected, since the genus *Adineta* subsumes vast cryptic diversity. *A. vaga* alone comprises at least 36 independently evolving entities (Fontaneto et al. 2011; Robeson et al. 2011). Rotifers of this genus have high dispersal potential (Fontaneto et al. 2008; Wilson & Sherman 2013) and the lifespan of a patch of lichen is short in evolutionary terms. If genetic exchange occurs so promiscuously among such diverse and mobile animals, it is remarkable that every donor species happened to be sampled in the same small area as the recipients at the same time. Even more striking, every case of incongruence involved a sequence that was also found natively in one of the other 81 rotifers

137 sampled, enabling the authors to construct a perfectly self-contained circular representation of the
138 "interspecific transfers" (their Figure 4).
139
140 We hypothesised that evidence interpreted as horizontal genetic exchanges might instead result
141 from accidental cross-contamination of rotifers or rotifer DNA between tubes during preparation of
142 samples. This would explain the hermetically self-referencing pattern of incongruent sequences in
143 samples showing "interspecific DNA transfers". Specifically, more than one animal (or loose DNA
144 from additional animals) may occasionally have been added to a single tube during the attempted
145 isolation of 576 individuals. It is technically challenging to isolate *Adineta* individually to Eppendorf
146 tubes, as we have found from personal experience (Supplemental Material). The animals are small
147 even when fully extended; when disturbed, they contract rapidly into tiny, motionless, transparent
148 spheroids that stick tenaciously to plasticware, and have about the same refractive index. If the
149 isolation protocol used by Debortoli et al. (2016) resulted in cross-contamination of a subset of
150 samples presented as individuals, it would give the misleading appearance of inter- and intraspecific
151 genetic exchanges. Here, we test this alternative hypothesis using two independent and
152 complementary sources of evidence: original Sanger sequencing chromatograms provided by the
153 authors, and alignments of genetic and genomic data from public repositories.
154
155 **Results and Discussion**
156
157 **Experimental determination of the effects of cross-contamination on Sanger chromatograms**
158
159 If samples showing "interspecific horizontal genetic transfers" contained DNA from two or more
160 animals of different species, additional haplotypes ought to be evident in chromatograms produced
161 by Sanger sequencing of PCR amplicons. In particular, prior sequencing of thousands of rotifers
162 indicates that each individual has only one mtCO1 haplotype (e.g. Fontaneto et al. 2009), therefore
163 to find double peaks in mtCO1 chromatograms would provide evidence for contamination.
164
165 We conducted an experiment to determine the pattern of mtCO1 chromatogram peaks when two
166 animals are present in one tube. We chose two bdelloid clones from our cultures: '*A. vaga* (AD008)',
167 which supplied the reference genome for *A. vaga* (Flot et al. 2013), and '*Adineta* sp. (AD006)'. We
168 prepared replicate Eppendorf tubes, either with a single individual, or deliberately contaminated
169 with two individuals, one from each species (Table S1). We extracted DNA and amplified the mtCO1

170 marker using the methods and primers described by Debortoli et al. (2016). Bidirectional
171 chromatograms were generated by direct Sanger sequencing with the PCR primers.
172
173 The phred quality scores of the chromatogram files (Ewing & Green 1998) were only slightly and not
174 significantly lower for tubes with two animals versus one (mean Q20: 90% vs. 92%, N = 38, t = 1.13, P
175 = 0.26). Even when two animals were present, the vast majority of base calls (97.9-99.6%) matched
176 a single species (Table S2). The additional animal did not manifest as obvious double peaks at the
177 expected polymorphic sites, but as small, subtle minority peaks, typically hidden in baseline noise
178 (Kronick 1997) and sometimes missing entirely. Perhaps this is unsurprising: double peaks are
179 seldom equal in height even when amplifying alleles from diploid heterozygotes (Kronick 1997). PCR
180 is exponential, and if two animals contribute divergent templates, large biases in final amplicon
181 representation may arise from small initial differences in numbers of cells or mitochondria, or the
182 efficiency of lysis, DNA extraction, primer binding, denaturation, etc. (Mullis et al. 1994).
183
184 We developed a simple quantitative method, called ConTAMPR (Contingency Table Analysis of
185 Minority Peak Ranks), to test whether the identity of the additional animal in deliberately
186 contaminated samples could be recovered from the pattern of minority peaks (Supplemental
187 Material). Briefly, chromatograms were aligned bidirectionally with the known sequences of both
188 *Adineta* clones. At each site where the two reference sequences differed, we manually ranked the
189 heights of the fluorescence trace lines corresponding to the three minority nucleotide bases, and
190 recorded the rank for the base fitting the known contaminant sequence. Using contingency table
191 analysis, we tested whether the distribution of peak height ranks differed from the expectation if
192 chromatogram noise were random. For additional rigor, we measured the distribution of minority
193 peaks fitted to a control species (*A. ricciae,* Segers & Shiel 2005) that was not present in any sample,
194 but whose sequence identity to both AD006 and AD008 at mtCO1 was equal (87.5%).
195
196 In uncontaminated single-animal samples with *A. sp*. (AD006), rank distributions of minority peaks
197 matching *A. vaga* (AD008) and *A. ricciae* did not differ significantly from the null expectation or from
198 each other (Figure 1A; χ^2 = 3.32, d.f. = 4, P = 0.51). In deliberately contaminated samples, however,
199 minority peaks fitted the known contaminant species significantly better than the null expectation
200 (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}).
201 ConTAMPR correctly detected that tubes contained two animals, identified the second sequence
202 and differentiated it from other candidates. Multiple biological and technical replicates yielded
203 similar results (Figure S1). This method works because PCR amplicons were directly sequenced,

leaving visual evidence of minority sequences. An approach based on cloning might miss minority sequences entirely (Mullis et al. 1994). The method is therefore suitable to test the directly sequenced amplicons of Debortoli et al. (2016) for contamination by multiple animals.

Evidence of cross-contamination in chromatograms generated by Debortoli et al. (2016)

To test for the signatures described above, we requested raw chromatogram files from Debortoli and colleagues. We obtained 36 out of at least 1152 mtCO1 files, including those for the six samples where interspecific transfers were claimed. We received 483 out of at least 656 chromatograms for 28S ribosomal DNA, and 133 out of at least 158 files for the EPIC25 (exon-primed intron-crossing) nuclear marker. We are grateful to the authors for their open and collaborative sharing of data.

Sample B11 was the first alphabetically to show "interspecific recombination". It was interpreted by Debortoli et al. as a single animal belonging to *A. vaga* Species A, with one mtCO1 haplotype (Hap6 [A]) and one 28S ribosomal sequence (Hap1 [A]). Other nuclear loci had supposedly acquired DNA horizontally from Species E (EPIC25 Hap35, EPIC63 Hap16 and Nu1054 Hap22). As discussed above, all three “transferred” haplotypes also were seen 'natively' in Species E 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 sequence comparable to Hap31 [E], which is found 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. = 2, P = 3.07 x 10^{-6}). We attempted to match the minority peaks to multiple control sequences, including the *A. vaga* reference genome and the other species delimited by Debortoli et al. (B, C, D, F). Hap31 [E] remained the best fit by far (Figure 2, χ^2= 39.2, d.f. = 12, P = 9.73 x 10^{-5}). Like other animals, single 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 sequences at the nuclear loci where interspecific genetic exchanges were claimed.

Although Debortoli et al. amplified mtCO1 directly from genomic DNA, they performed PCR for the 28S, EPIC25, EPIC63 and Nu1054 nuclear markers after whole-genome amplification (WGA) of the

238 samples via an Illustra GenomiPhi V2 DNA Amplification Kit (GE Healthcare, Amersham Biosciences).
239 When DNA from two animals was present, the two mtCO1 haplotypes were represented very
240 unequally among our PCR amplicons (Figure 1B). Interposing another nonlinear amplification step
241 introduces further opportunities for bias: some sequences might be dropped entirely, or a majority
242 of amplicons might be generated from a minority template. This particular kit shows a bias in favor
243 of templates with lower guanine-cytosine (GC) content (Han et al. 2012), which we suggest as an
244 important factor in selective loss of alleles (Supplemental Material). For these reasons, it was less
245 clear whether nuclear loci would show corresponding evidence of a second animal. However, we
246 attempted to conduct the same analysis, first at the ribosomal 28S marker. The putative second
247 animal was predicted to show a 28S sequence characteristic of Species E.
248
249 As predicted, the fit of minority peaks to Species E was significantly better than the null expectation
250 (Figure 3), and also superior to Species F (χ^2=22.45, d.f. = 2, P = 1.3 x 10^{-5}), B (χ^2=12.75, d.f. = 2, P =
251 0.0017), 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 results
252 remain significant even after a conservative Bonferroni correction (α =0.0083). Species E was a
253 better fit than Species D and the *A. vaga* reference genome too, but not significantly so, as the three
254 species are nearly identical at the conserved 28S marker. The fit to minority peaks recapitulates a
255 phylogenetic tree based on 28S (Figure 3): species more closely related to E fit better. The evidence
256 at 28S therefore reinforces the mtCO1 results, indicating a second animal belonging to Species E.
257 Because Debortoli et al. already reported Species E alleles at EPIC25, EPIC63 and Nu1054, evidence
258 from all five sequenced loci is now brought into congruence, as predicted if Sample B11 contained
259 an animal belonging to Species E, with a similar genotype to Individual 81.
260
261 **Evidence for "interspecific genetic exchanges" is explained by heterospecific cross-contamination**
262
263 We used the same methods to investigate the other five samples where Debortoli et al. diagnosed
264 "interspecific horizontal genetic transfers" based on incongruent species assignment among loci.
265 Where the relevant chromatograms were provided, we aligned them bidirectionally against the
266 sequences reported by Debortoli et al., then analysed the pattern of minority peaks with reference
267 to the sequences of other species reported in the study (Supplemental Material).
268
269 All six samples showed significant evidence of additional sequences that were not reported by
270 Debortoli et al. (Table 1). Three samples (B11, B22, B39) each contained two clearly identifiable
271 mtCO1 haplotypes, which is only consistent with DNA from two animals. Even if mtDNA itself could

272 undergo interspecific horizontal transfer (as Debortoli et al. imply for Sample B39), intergenerational
273 bottlenecking of mitogenomes (Mishra & Chan 2014) would still result in a single mtCO1 haplotype
274 (J.-F. Flot, pers. comm). Samples B14 and B3B1 showed evidence of DNA originating in three
275 different rotifers (from species A, C and E). The mtCO1 chromatograms for these samples were
276 extreme outliers in phred quality scores (Figure 4), with too much noise to narrow down just one
277 candidate contaminant (Table 1; Figure S7).
278
279 In samples B11, B14 and B22, additional 28S sequences were found. This is not an expected result of
280 HGT, because ribosomal DNA undergoes concerted evolution (Liao 2000) that is believed to preclude
281 the maintenance of substantially divergent 28S copies in a single rotifer, even if one arrived via HGT
282 (J.-F. Flot, N. Debortoli & K. Van Doninck, pers. comm.). Ribosomal DNA has been amplified from
283 hundreds of rotifers without finding copies that differ by more than 2-3 bases (e.g. Tang et al. 2012).
284 Debortoli et al. found no more than three differences between 28S copies in any of 82 animals (their
285 Figure 3A). In contrast, the two sequences we found in Samples B14 and B22 differ at over 40
286 positions in the first 700bp fragment alone. As a consequence of multiple peaks from additional
287 sequences, quality scores for 28S chromatograms were significantly lower where transfer was
288 claimed than where it was not (Figure 5; Mann-Whitney Test: $N = 122$, $W = 373.5$, $P = 0.015$).
289
290 In Samples B39 and D14, we recovered the 'native' sequences that were supposed to have been
291 replaced by horizontal acquisitions. All loci in both samples are now brought into congruence. A
292 parsimonious inference is that they represent typical animals with haplotype associations similar to
293 conspecifics. The incongruent sequences arise from WGA or PCR of contaminating DNA fragments,
294 or from a second animal whose alleles were dropped during amplification at other loci. Consistent
295 with biased or capricious amplification, the predicted 'native' sequence in Sample D14 was almost
296 equivalent in peak strength to the heterospecific sequence after an initial PCR, but absent among
297 amplicons from a replicate PCR using the same tube of template (Supplemental Material). If the
298 locus had only been amplified once, there might have been no evidence of the native sequence, and
299 the case for interspecific recombination would have been harder to reject. A lack of minority peaks
300 after this replicate PCR confirms an important message in this dataset: even when a sample contains
301 two different sequences, one may be dropped during any given WGA or PCR. A significant pattern of
302 minority peaks is thus sufficient but not necessary to indicate a contaminating sequence, whereas
303 the absence of such a pattern is necessary but not sufficient to exclude it.
304
305

306 **“Interspecific recombination” is not mechanistically compatible with genomic evidence**

307

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

315

316 In the context of HGT, Thomas and Nielsen (2005) define HR as "recombination that depends on
317 extensive segments of high sequence similarity between two DNA molecules" (p. 714). As Debortoli
318 et al. themselves remark, the frequency of HR "is strongly correlated with the degree of identity
319 between the recombining DNA fragments and dramatically declines as the sequences diverge." A
320 log-linear decline in HR with decreasing sequence identity is well established in bacteria (Watt et al.
321 1985; Zawadzki et al. 1995; Vulić et al. 1997; Majewski 2001; Thomas & Nielsen 2005); protists (Bell
322 & McCulloch 2003); yeast (Datta et al. 1997); plants (Opperman et al. 2004; Li et al. 2006) and
323 animals (LaRocque & Jasin 2010; Do & LaRocque 2015). Datta et al. (1997) found that a single
324 mismatched base (99.7% identity) reduced HR rates fourfold in yeast, and reductions to 99% or 90%
325 identity reduced HR rates by one and two orders of magnitude respectively. At these distances, the
326 relationship is largely governed by active mismatch repair systems, and HR does not decline so
327 dramatically if these are abolished. However, when sequence identity falls below 90%, rates of HR
328 decline exponentially even in mutants devoid of mismatch repair, indicating that the machinery of
329 HR itself fails to engage when so many bases are mispaired. At 83.5% identity, HR is effectively
330 absent in yeast (three recombinant cells per billion: Datta et al. 1997), and rates were too low to
331 measure even for otherwise promiscuous *Bacillus* species (Zawadzki et al. 1995).

332

333 Following this logic, Debortoli et al. remark that "closely related species should be more prone to
334 genetic exchanges, which would explain why our study, focusing on intrageneric variation within the
335 morphospecies *A. vaga*, detected multiple cases of genetic transfers." Indeed, an extremely high
336 frequency of HR is required to explain their data. Even though only five loci were examined, nearly
337 10% of the sampled "individuals" were claimed as interspecific recombinants. To accommodate the
338 self-contained pattern of transfers, these events must be so recent that putative donor species and
339 recipient clones were all still living together at the time of the study, with transferred sequences

340 remaining identical between them. Moreover, HR must have occurred at least twice in each case:
341 once when the exogenous DNA was first integrated, and again when "gene conversion promptly
342 copied the integrated DNA on its homologous region". Such high frequencies of homologous
343 exchange are possible only if donor and recipient sequences have very high sequence identity.
344
345 We tested this prediction for each case of "interspecific recombination" using a simple method. We
346 aligned each incongruent sequence from a putative donor species against the native sequence it was
347 supposed to have replaced, and measured pairwise identities (Supplemental Material). This
348 recreates the genetic divergences across which Debortoli et al. posit multiple, recent HR events
349 (Table 2). The mean identity between sequences is just 74.8% (median 72.3%). This surprisingly low
350 figure was validated for a genomic region of approximately 10kb around the 400bp EPIC25 intronic
351 marker (Table S3, S4). Identity values are the same or lower regardless whether we consider exons,
352 introns or intergenic regions. These numbers are not compatible with current understanding of HR
353 in the model systems discussed above, or with current views of homologous transfer in the genome
354 of *A. vaga*. The assembly of Flot et al. (2013) indicates that gene conversion and concerted
355 evolution occur between sequence pairs that "are on average 96.2% identical at the nucleotide level
356 (median = 98.6%)." Debortoli et al. (2016) claim interspecific recombination between sequences
357 approximately an order of magnitude more divergent.
358
359 The tetraploid genome of *A. vaga* supplies a second critical benchmark: each homologous pair of
360 genes has a pair of 'ohnologous' copies whose mean identity is "73.6% (median = 75.1%)" (Flot et al.
361 2013). This is very close to the value we calculated for "interspecific horizontal genetic transfers",
362 yet ohnologs have been evolving independently for millions of years within the same genomes, cells,
363 individuals and clones, with "no conspicuous tracts of identity" between them (Hur et al. 2009). This
364 feature of *A. vaga* militates strongly against the claim that molecules with 74.8% sequence identity
365 could undergo frequent recombination or gene conversion within this genome, still less horizontally.
366
367 We considered the possibility that interspecific recombination might involve some alternative
368 mechanism with less stringent identity requirements than HR. For instance, microhomology-
369 mediated end joining involves “the use of 5-25 bp microhomologous sequences during the
370 alignment of broken ends before joining" (McVey & Lee 2008). We measured the degree of
371 microhomology between two "transferred" EPIC25 regions at scales from 1-40bp, but found they
372 were no more similar than 7650 independently evolving ohnologous pairs in the same genome, in
373 close agreement with comparisons based on global homology (Figure S9). One sequence even

374 shared more microhomology with its own ohnolog (the EPIC63 region) than with its "transferred"
375 partner. This excludes the hypothesis that microhomology-based mechanisms mediate interspecific
376 HGT. If that were so, ohnologous loci could not evolve independently, as they share at least as much
377 microhomology at every scale, and their DNA must be more abundant and accessible in any genome,
378 cell or individual than DNA arriving across horizontal barriers.
379
380 Integration of DNA over large genetic distances must be possible at least occasionally in bdelloid
381 rotifers, since genes with little or no homology to metazoan sequences are incorporated (Gladyshev
382 et al. 2008). However, the mechanism in these cases would not require homologous substitution,
383 and therefore would not prevent ohnologs evolving independently. It may involve a combination of
384 individually unlikely events, which would explain why absolute rates appear low (Eyres et al. 2015).
385
386 **Evidence for "intraspecific horizontal exchanges" is explained by conspecific cross-contamination**
387
388 Chromatogram analysis revealed that at least six samples interpreted as individuals by Debortoli et
389 al. (2016) were contaminated with DNA from two or three animals belonging to different species.
390 Elsewhere in the study, the authors use the same dataset to claim evidence for "intraspecific DNA
391 exchanges". It seems prudent to evaluate the alternative hypothesis that these cases correspond to
392 samples that were contaminated with DNA from two animals belonging to the same species.
393
394 Debortoli et al. report three trios of "heterozygous individuals" in Species A and C that seem to share
395 haplotypes "in a cyclic fashion...(a||b), (b||c), and (c||a)". The term 'heterozygous' is not strictly
396 appropriate without segregation, but we follow this usage for simplicity. The authors claim that this
397 pattern "can only be explained by recombination between individuals", but an alternative possibility
398 is that one or more tubes per trio were contaminated with DNA from a second conspecific animal.
399 For example, two tubes might each contain one heterozygote: (a||b) and (b||c), while a third
400 contains DNA from two animals homozygous at the focal locus: (c||c) & (a||a). As the authors
401 rightly remark, this requires no intraspecific horizontal exchange, since "the observation of three
402 distinct haplotypes a, b, and c in heterozygous individuals with genotypes (a||b) and (b||c) may be
403 explained by mutations and gene conversions alone". At least six other permutations of conspecific
404 contamination are capable of producing a haplotype trio in the same way (Table S5).
405
406 Unlike heterospecific contamination, conspecific contamination cannot be tested via chromatograms.
407 Samples in Species A and C all share the same one or two 28S copies, and identical mtCO1 sequences

408 are seen in animals that are homo- or heterozygous for EPIC25 haplotypes in the trios, rendering the
409 DNA of two conspecific rotifers indistinguishable at these loci. Instead, we can apply probability
410 theory. Evidence for inter- and intraspecific transfer relies on the same 576 tubes prepared from the
411 same natural material. Given the relative abundances of *Adineta* species in that material, and the
412 number of demonstrated heterospecific contamination events, we can calculate the number of tubes
413 expected to be contaminated with DNA from animals belonging to the same species (Table S6). Even
414 using highly conservative calculations, eight out of 82 tubes (95% CI: 4-15) are expected to be
415 contaminated in this way. Just three such tubes would be sufficient to explain the evidence for
416 intraspecific allele sharing; we calculate a 99.27% probability that conspecific contamination events
417 occurred at the minimum rate required to generate three cyclic trio artefacts.
418
419 Given its high frequency, Species C is by far the most likely to show conspecific contamination. The
420 number of tubes expected to contain DNA from two Species C rotifers is 7 (95% CI: 4-14). Debortoli
421 et al. present genomic data for three samples interpreted as Species C individuals, selected for
422 sequencing *post-hoc* because one PCR marker seemed to show conspecific allele-sharing. Given the
423 calculations described above, the probability is 99.98% that one or more tubes contained DNA from
424 two animals belonging to Species C (Supplemental Material). Contamination of this sort would also
425 explain why the same Species C trio seemed to show "signatures of inter-individual recombination"
426 at unlinked loci (EPIC63, *hsp82*) even though such concordance is not expected if DNA fragments at
427 different loci undergo recombination and gene conversion frequently and independently.
428
429 The issue of transfer across non-credible genetic distances does not affect purported intraspecific
430 recombination, since mean homology between sequences in trios is 99.1%. Instead, the opposite
431 problem applies: when sequences differ at just 3-5 sites, "allele sharing" may need no explanation
432 beyond "mutations and gene conversions alone", as Debortoli et al. rightly note. We tested this
433 hypothesis for the two trios in Species A by analysing shared polymorphisms (Figure S10). In both
434 cases, a single point mutation and a single gene conversion event are sufficient to explain the whole
435 cycle. For these two trios, it is not even necessary to invoke the high probabilities of conspecific
436 cross-contamination calculated above, let alone intraspecific horizontal exchanges.
437
438 **Conclusions**
439
440 Debortoli et al. (2016) claimed to supply "strong evidence that inter- and intraspecific DNA exchanges
441 occur within the bdelloid rotifer genus *Adineta*." However, chromatograms provided by the authors

442 reveal that samples treated as individuals were contaminated with DNA from multiple rotifer species,
443 whose identities can be recovered statistically. The probability of these patterns emerging from
444 random noise is vanishingly low. Accidental cross-contamination parsimoniously explains the results
445 of Debortoli et al. without reference to extraordinary or novel genetic phenomena. We can reject
446 the argument that some transfers are real and others artefacts, since genetic alignments show that
447 interspecific recombination is not a biologically credible interpretation for any of the incongruent
448 sequences reported. Pairwise identities are not compatible with homologous substitution even
449 within the *A. vaga* genome, still less with HGT at the rate the dataset implies. Given the observed
450 rate of heterospecific contamination, the probability exceeds 99.9% that conspecific contamination
451 occurred sufficiently often to explain evidence for intraspecific DNA exchanges too, where any
452 explanation is needed beyond mutations and gene conversions alone. In our view, these analyses
453 constitute clear evidence that the findings of Debortoli et al. (2016) are unreliable. The interesting
454 hypothesis that "bdelloid individuals of the genus *Adineta* exchange DNA within and between
455 species" may be true or false, but this work supplies no credible evidence to address that question.
456
457 The study appears to have met with wide acceptance (Krause et al. 2016; Ram & Hadany 2016; Sharp
458 & Otto 2016; Tilquin & Kokko 2016), perhaps because bdelloid asexuality has long been considered
459 problematic (Maynard Smith 1986), and the work seemed to confirm the enticing idea that sex is
460 "replaced" by HGT (Gladyshev et al. 2008; Flot et al. 2013). The high proportion of foreign genes in
461 bdelloid genomes lends this hypothesis a superficial appeal, but current formulations are simplistic
462 and imprecise, and a clearer theoretical framework is needed to assess its evolutionary plausibility.
463 In particular, sex has genome-wide consequences each generation via segregation, crossing-over,
464 independent assortment, outcrossing and sexual selection. These underpin a plethora of formal
465 models for the maintenance of amphimixis (Kondrashov 1993). Fragmentary horizontal transfer,
466 homologous or otherwise, is not a simple analog of meiotic sex, but has quite different population
467 genetic consequences (Redfield 2001; Narra & Ochman 2006; Agrawal 2009; Croucher et al. 2016).
468
469 The work of Debortoli et al. (2016) was criticised by Signorovitch et al. (2016), who did not identify
470 the issues discussed here, but were concerned that the results seemed incompatible with their own
471 prior claim of "a striking pattern of allele sharing consistent with sexual reproduction and with
472 meiosis of an atypical sort" in the bdelloid *Macrotrachela quadricornifera* (Signorovitch et al. 2015).
473 As argued by Debortoli et al., "our observations do not support the hypothesis of an *Oenothera*-like
474 meiosis" in *Adineta,* because "among the 82 *A. vaga* individuals sequenced for four nuclear markers,
475 no trio of individuals presented congruent patterns of shared sequences" (Flot et al. 2016). This lack

476 of evidence for congruent allele sharing cannot be attributed to cross-contamination, which would
477 not obscure a pattern so striking and widespread as that reported by Signorovitch et al. (2015). That
478 study itself features some arguments and patterns that seem to require empirical clarification, and
479 we join the authors in "awaiting full genome sequencing of the allele-sharing individuals of *M.*
480 *quadricornifera*" (Signorovitch et al. 2016), which will shed further light.
481
482 Schwander (2016) commented on the work of Signorovitch et al. (2015) and Debortoli et al. (2016),
483 under the title "*The End of an Ancient Asexual Scandal*". Schwander asserts that "these two studies
484 show beyond doubt that genetic exchange between individuals occurs in different bdelloid species."
485 Schwander takes the position that "even small amounts of recombination and genetic exchange
486 between individuals appear to be enough to provide all the benefits of sex", and therefore judges
487 that "bdelloids should no longer be considered as asexuals." With the advantage of hindsight, we
488 suggest some of these rather strong assertions might now be considered premature.
489
490 In the words of Maynard Smith (1986), bdelloid rotifers "remain something of an evolutionary
491 scandal." Molecular inquiries have revealed some truly extraordinary features in these tiny and
492 unassuming creatures (Arkhipova & Meselson 2000; Gladyshev et al. 2008; Mark Welch et al. 2008;
493 Boschetti et al. 2012; Hespeels et al. 2014; Arkhipova et al. 2017), inviting speculation about links to
494 their unusual reproductive mode. It is tempting to seek confirmation of these exciting ideas, and to
495 expect further extraordinary discoveries. However, that approach opens the door to well-known
496 biases, both when interpreting data and when evaluating the work of others (Nickerson 1998).
497 Given concerns around contamination and misinterpretation, we join others in recommending that
498 heightened scrutiny be applied to future claims of 'non-canonical' genetic exchange in microscopic
499 animals (Richards & Monier 2016), and to work on the molecular genetics of bdelloid rotifers in
500 particular. A more cautious and incremental programme will better facilitate firm progress, and help
501 exploit the unusual leverage that bdelloids offer on fundamental evolutionary and genetic questions.
502
503 **Acknowledgements**
504
505 We sincerely thank N. Debortoli, K. Van Doninck and J.-F. Flot for freely sharing chromatograms to
506 help us test the alternative hypothesis we had proposed. This goes beyond standard practice for
507 sharing sequencing data, which perhaps ought to be revisited. We appreciate their transparent and
508 scholarly conduct, and their thoughtful and collegial correspondence since we first communicated
509 our concerns in April 2016. We are grateful to M. Blaxter, G. Koutsovoulos and M. Meselson for

helpful comments, and to P. MacMahon for mathematical advice. 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.

Author Contributions

Conceptualization: C.G.W.; Methodology: C.G.W. and R.W.N.; Software: R.W.N.; Formal Analysis: C.G.W. and T.B.; Investigation: C.G.W.; Data Curation: C.G.W.; Writing- Original Draft: C.G.W.; Writing- Review & Editing: R.W.N., T.B. and C.G.W.; Visualization: C.G.W. and R.W.N.; Supervision: C.G.W. and T.B.; Project Administration: C.G.W. and T.B.; Funding Acquisition: C.G.W. and T.B.

References

1. Agrawal, A.F. (2009). Spatial heterogeneity and the evolution of sex in diploids. *Am. Nat.*, 174, S54–S70

2. Arkhipova, I. & Meselson, M. (2000). Transposable elements in sexual and ancient asexual taxa. *Proc. Natl. Acad. Sci. U. S. A.*, 97, 14473–7

3. Arkhipova, I.R., Yushenova, I.A. & Rodriguez, F. (2017). Giant reverse transcriptase-encoding transposable elements at telomeres. *Mol. Biol. Evol.*

4. Bell, G. (1982). *The masterpiece of nature: the evolution and genetics of sexuality*. Croom Helm, London

5. Bell, J.S. & McCulloch, R. (2003). Mismatch repair regulates homologous recombination, but has little influence on antigenic variation, in *Trypanosoma brucei*. *J. Biol. Chem.*, 278, 45182–45188

6. Birky, C.W. (1996). Heterozygosity, heteromorphy, and phylogenetic trees in asexual eukaryotes. *Genetics*, 144, 427–437

7. Birky, C.W. (2010). Positively negative evidence for asexuality. *J. Hered.*, 101 Suppl, S42-5

8. Boothby, T.C., Tenlen, J.R., Smith, F.W., Wang, J.R., Patanella, K.A., Osborne Nishimura, E., *et al.* (2015). Evidence for extensive horizontal gene transfer from the draft genome of a tardigrade. *Proc. Natl. Acad. Sci.*, 112, 15976–15981

545

546 9. Boschetti, C., Carr, A., Crisp, A., Eyres, I., Wang-Koh, Y., Lubzens, E., *et al.* (2012). Biochemical
547 diversification through foreign gene expression in bdelloid rotifers. *PLoS Genet.*, 8, e1003035
548

549 10. Burt, A. (2000). Sex, recombination, and the efficacy of selection—Was Weismann right?
550 *Evolution*, 54, 337–351
551

552 11. Crisp, A., Boschetti, C., Perry, M., Tunnacliffe, A. & Micklem, G. (2015). Expression of multiple
553 horizontally acquired genes is a hallmark of both vertebrate and invertebrate genomes. *Genome*
554 *Biol.*, 16
555

556 12. Croucher, N.J., Mostowy, R., Wymant, C., Turner, P., Bentley, S.D. & Fraser, C. (2016). Horizontal
557 DNA transfer mechanisms of bacteria as weapons of intragenomic conflict. *PLoS Biol.*, 14, 1–42
558

559 13. Datta, A., Hendrix, M., Lipsitch, M. & Jinks-Robertson, S. (1997). Dual roles for DNA sequence
560 identity and the mismatch repair system in the regulation of mitotic crossing-over in yeast. *Proc.*
561 *Natl. Acad. Sci. U. S. A.*, 94, 9757–62
562

563 14. Debortoli, N., Li, X., Eyres, I., Fontaneto, D., Hespeels, B., Tang, C.Q., *et al.* (2016). Genetic
564 exchange among bdelloid rotifers is more likely due to horizontal gene transfer than to meiotic sex.
565 *Curr. Biol.*, 26, 723–732
566

567 15. Delmont, T.O. & Eren, A.M. (2016). Identifying contamination with advanced visualization and
568 analysis practices: metagenomic approaches for eukaryotic genome assemblies. *PeerJ*, 4, e1839
569

570 16. Do, A.T. & LaRocque, J.R. (2015). The role of *Drosophila* mismatch repair in suppressing
571 recombination between diverged sequences. *Sci. Rep.*, 5, 17601
572

573 17. Ewing, B. & Green, P. (1998). Base-calling of automated sequencer traces using phred. II. Error
574 probabilities. *Genome Res.*, 8, 186–194
575

576 18. Eyres, I., Boschetti, C., Crisp, A., Smith, T.P., Fontaneto, D., Tunnacliffe, A., *et al.* (2015).
577 Horizontal gene transfer in bdelloid rotifers is ancient, ongoing and more frequent in species from
578 desiccating habitats. *BMC Biol.*, 13, 90

579

580 19. Flot, J., Debortoli, N., Hallet, B. & Van Doninck, K. (2016). Response to Signorovitch et al. *Curr.*
581 *Biol.*, 26, R739–R755
582

583 20. Flot, J., Hespeels, B., Li, X., Noel, B., Arkhipova, I., Danchin, E.G.J., *et al.* (2013). Genomic
584 evidence for ameiotic evolution in the bdelloid rotifer *Adineta vaga*. *Nature*, 500, 453–457
585

586 21. Fontaneto, D., Barraclough, T.G., Chen, K., Ricci, C. & Herniou, E. a. (2008). Molecular evidence
587 for broad-scale distributions in bdelloid rotifers: everything is not everywhere but most things are
588 very widespread. *Mol. Ecol.*, 17, 3136–46
589

590 22. Fontaneto, D., Iakovenko, N., Eyres, I., Kaya, M., Wyman, M. & Barraclough, T.G. (2011). Cryptic
591 diversity in the genus *Adineta* Hudson & Gosse, 1886 (Rotifera: Bdelloidea: Adinetidae): A DNA
592 taxonomy approach. *Hydrobiologia*, 662, 27–33
593

594 23. Fontaneto, D., Kaya, M., Herniou, E. a & Barraclough, T.G. (2009). Extreme levels of hidden
595 diversity in microscopic animals (Rotifera) revealed by DNA taxonomy. *Mol. Phylogenet. Evol.*, 53,
596 182–9
597

598 24. Gladyshev, E. a., Meselson, M. & Arkhipova, I.R. (2008). Massive horizontal gene transfer in
599 bdelloid rotifers. *Science*, 320, 1210–1213
600

601 25. Gladyshev, E. & Meselson, M. (2008). Extreme resistance of bdelloid rotifers to ionizing
602 radiation. *Proc. Natl. Acad. Sci. U. S. A.*, 105, 5139–44
603

604 26. Han, T., Chang, C.-W., Kwekel, J.C., Chen, Y., Ge, Y., Martinez-Murillo, F., *et al.* (2012).
605 Characterization of whole genome amplified (WGA) DNA for use in genotyping assay development.
606 *BMC Genomics*, 13, 217
607

608 27. Hartfield, M. & Keightley, P.D. (2012). Current hypotheses for the evolution of sex and
609 recombination. *Integr. Zool.*, 7, 192–209
610

611 28. Hashimoto, T., Horikawa, D.D., Saito, Y., Kuwahara, H., Kozuka-Hata, H., Shin-I, T., *et al.* (2016).
612 Extremotolerant tardigrade genome and improved radiotolerance of human cultured cells by

613 tardigrade-unique protein. *Nat. Commun.*, 7, 12808
614
615 29. Hebert, P.D.N., Cywinska, A., Ball, S.L. & deWaard, J.R. (2003). Biological identifications through
616 DNA barcodes. *Proc. R. Soc. B Biol. Sci.*, 270, 313–321
617
618 30. Hespeels, B., Knapen, M., Hanot-Mambres, D., Heuskin, A.C., Pineux, F., Lucas, S., *et al.* (2014).
619 Gateway to genetic exchange? DNA double-strand breaks in the bdelloid rotifer *Adineta vaga*
620 submitted to desiccation. *J. Evol. Biol.*, 27, 1334–1345
621
622 31. Hickey, D.A. (1982). Selfish DNA: a sexually-transmitted nuclear parasite. *Genetics*, 101, 519–531
623
624 32. Hsu, W.S. (1956). Oogenesis in the Bdelloidea rotifer *Philodina roseola* Ehrenberg. *Biol. Bull.*, 57,
625 283–296
626
627 33. Hudson, C.T. & Gosse, P.H. (1886). *The Rotifera; or Wheel-Animalcules*. Longmans, Geen & Co.,
628 London
629
630 34. Hur, J.H., Van Doninck, K., Mandigo, M.L. & Meselson, M. (2009). Degenerate tetraploidy was
631 established before bdelloid rotifer families diverged. *Mol. Biol. Evol.*, 26, 375–83
632
633 35. Judson, O.P. & Normark, B.B. (1996). Ancient asexual scandals. *Trends Ecol. Evol.*, 11, 41–6
634
635 36. Kondrashov, A.S. (1993). Classification of hypotheses on the advantage of amphimixis. *J. Hered.*,
636 84, 372–87
637
638 37. Koutsovoulos, G., Kumar, S., Laetsch, D.R., Stevens, L., Daub, J., Conlon, C., *et al.* (2016). No
639 evidence for extensive horizontal gene transfer in the genome of the tardigrade *Hypsibius dujardini*.
640 *Proc. Natl. Acad. Sci.*, 113, 5053–5058
641
642 38. Krause, A., Pachl, P., Schulz, G., Lehmitz, R., Seniczak, A., Schaefer, I., *et al.* (2016). Convergent
643 evolution of aquatic life by sexual and parthenogenetic oribatid mites. *Exp. Appl. Acarol.*, 70, 439–
644 453
645
646 39. Kronick, M.N. (1997). Heterozygote determination using automated DNA sequencing

647 technology. In: *Laboratory Methods for the Detection of Mutations and Polymorphisms in DNA* (ed.
648 Taylor, G.R.). CRC Press, pp. 175–190
649
650 40. LaRocque, J.R. & Jasin, M. (2010). Mechanisms of recombination between diverged sequences in
651 wild-type and BLM-deficient mouse and human cells. *Mol. Cell. Biol.*, 30, 1887–1897
652
653 41. Lehtonen, J., Jennions, M.D. & Kokko, H. (2012). The many costs of sex. *Trends Ecol. Evol.*, 27,
654 172–178
655
656 42. Li, L., Jean, M. & Belzile, F. (2006). The impact of sequence divergence and DNA mismatch repair
657 on homeologous recombination in *Arabidopsis*. *Plant J.*, 45, 908–916
658
659 43. Liao, D. (2000). Gene conversion drives within genic sequences: concerted evolution of
660 ribosomal RNA genes in bacteria and archaea. *J. Mol. Evol.*, 51, 305–17
661
662 44. Lunt, D.H. (2008). Genetic tests of ancient asexuality in root knot nematodes reveal recent
663 hybrid origins. *BMC Evol. Biol.*, 8, 194
664
665 45. Majewski, J. (2001). Sexual isolation in bacteria. *FEMS Microbiol. Lett.*, 199, 161–169
666
667 46. Mark Welch, D. & Meselson, M. (2000). Evidence for the evolution of bdelloid rotifers without
668 sexual reproduction or genetic exchange. *Science*, 288, 1211–5
669
670 47. Mark Welch, D.B., Mark Welch, J.L. & Meselson, M. (2008). Evidence for degenerate tetraploidy
671 in bdelloid rotifers. *Proc. Natl. Acad. Sci. U. S. A.*, 105, 5145–9
672
673 48. Mark Welch, D.B., Ricci, C. & Meselson, M. (2009). Bdelloid rotifers: progress in understanding
674 the success of an evolutionary scandal. In: *Lost Sex* (eds. Schön, I., Martens, K. & Dijk, P.). Springer
675 Netherlands, Dordrecht, pp. 259–279
676
677 49. Mark Welch, J.L. & Meselson, M. (1998). Karyotypes of bdelloid rotifers from three families.
678 *Hydrobiologia*, 387, 403–407
679
680 50. Maynard Smith, J. (1978). *The Evolution of Sex*. Cambridge University Press, Cambridge

681

682 51. Maynard Smith, J. (1986). Contemplating life without sex. *Nature*, 324, 300–301

683

684 52. McVey, M. & Lee, S.E. (2008). MMEJ repair of double-strand breaks (director's cut): deleted
685 sequences and alternative endings. *Trends Genet.*, 24, 529–538

686

687 53. Meirmans, S., Meirmans, P.G. & Kirkendall, L.R. (2012). The costs of sex: facing real-world
688 complexities. *Q. Rev. Biol.*, 87, 19–40

689

690 54. Mishra, P. & Chan, D.C. (2014). Mitochondrial dynamics and inheritance during cell division,
691 development and disease. *Nat. Rev. Mol. Cell Biol.*, 15, 634–646

692

693 55. Mullis, K.B., Ferre, F. & Gibbs, R.A. (1994). *The polymerase chain reaction*. Birkhäuser, Boston

694

695 56. Narra, H.P. & Ochman, H. (2006). Of what use is sex to bacteria? *Curr. Biol.*, 16, 705–710

696

697 57. Nickerson, R.S. (1998). Confirmation bias: A ubiquitous phenomenon in many guises. *Rev. Gen.*
698 *Psychol.*, 2, 175–220

699

700 58. Normark, B.B., Judson, O.P. & Moran, N.A. (2003). Genomic signatures of ancient asexual
701 lineages. *Biol. J. Linn. Soc.*, 79, 69–84

702

703 59. Opperman, R., Emmanuel, E. & Levy, A.A. (2004). The effect of sequence divergence on
704 recombination between direct repeats in arabidopsis. *Genetics*, 168, 2207–2215

705

706 60. Palmer, S.C. & Norton, R.A. (1991). Taxonomic, geographic and seasonal distribution of
707 thelytokous parthenogenesis in the Desmonomata (Acari: Oribatida). *Exp. Appl. Acarol.*, 12, 67–81

708

709 61. Pons, J., Barraclough, T., Gomez-Zurita, J., Cardoso, A., Duran, D., Hazell, S., *et al.* (2006).
710 Sequence-Based Species Delimitation for the DNA Taxonomy of Undescribed Insects. *Syst. Biol.*, 55,
711 595–609

712

713 62. Pouchkina-Stantcheva, N.N., McGee, B.M., Boschetti, C., Tolleter, D., Chakrabortee, S., Popova,
714 A. V, *et al.* (2007). Functional divergence of former alleles in an ancient asexual invertebrate.

Science, 318, 268–71

63. Ram, Y. & Hadany, L. (2016). Condition-dependent sex: who does it, when and why? *Philos. Trans. R. Soc. B Biol. Sci.*, 371, 20150539

64. Redfield, R.J. (2001). Do bacteria have sex? *Nat. Rev. Genet.*, 2, 634–639

65. Ricci, C.N. (1987). Ecology of bdelloids: how to be successful. *Hydrobiologia*, 147, 117–127

66. Richards, T.A. & Monier, A. (2016). A tale of two tardigrades. *Proc. Natl. Acad. Sci.*, 113, 4892–4894

67. Robeson, M.S., King, a. J., Freeman, K.R., Birky, C.W., Martin, a. P. & Schmidt, S.K. (2011). Soil rotifer communities are extremely diverse globally but spatially autocorrelated locally. *Proc. Natl. Acad. Sci.*, 1–5

68. Schurko, A.M., Neiman, M. & Logsdon, J.M. (2009). Signs of sex: what we know and how we know it. *Trends Ecol. Evol.*, 24, 208–17

69. Schwander, T. (2016). Evolution: the end of an ancient asexual scandal. *Curr. Biol.*, 26, R233–R235

70. Schwander, T., Crespi, B.J., Gries, R. & Gries, G. (2013). Neutral and selection-driven decay of sexual traits in asexual stick insects. *Proc. R. Soc. B*, 280, 20130823

71. Segers, H. (2007). Annotated checklist of the rotifers (Phylum Rotifera), with notes on nomenclature, taxonomy and distribution. *Zootaxa*, 1564, 1–104

72. Segers, H. & Shiel, R.J. (2005). Tale of a sleeping beauty: a new and easily cultured model organism for experimental studies on bdelloid rotifers. *Hydrobiologia*, 546, 141–145

73. Sharp, N.P. & Otto, S.P. (2016). Evolution of sex: Using experimental genomics to select among competing theories. *BioEssays*, 38, 751–757

749 74. Signorovitch, A., Hur, J., Gladyshev, E. & Meselson, M. (2015). Allele sharing and evidence for
750 sexuality in a mitochondrial clade of bdelloid rotifers. *Genetics*, 200, 581–90
751
752 75. Signorovitch, A., Hur, J., Gladyshev, E. & Meselson, M. (2016). Evidence for meiotic sex in
753 bdelloid rotifers. *Curr. Biol.*, 26, R754–R755
754
755 76. Smith, R.J., Kamiya, T. & Horne, D.J. (2006). Living males of the "ancient asexual" Darwinulidae
756 (Ostracoda: Crustacea). *Proc. R. Soc. B*, 273, 1569–78
757
758 77. Speijer, D., Lukeš, J. & Eliáš, M. (2015). Sex is a ubiquitous, ancient, and inherent attribute of
759 eukaryotic life. *Proc. Natl. Acad. Sci.*, 112, 8827–8834
760
761 78. Tang, C.Q., Leasi, F., Obertegger, U., Kieneke, A., Barraclough, T.G. & Fontaneto, D. (2012). The
762 widely used small subunit 18S rDNA molecule greatly underestimates true diversity in biodiversity
763 surveys of the meiofauna. *Proc. Natl. Acad. Sci.*, 109, 16208–16212
764
765 79. Thomas, C.M. & Nielsen, K.M. (2005). Mechanisms of, and barriers to, horizontal gene transfer
766 between bacteria. *Nat. Rev. Microbiol.*, 3, 711–721
767
768 80. Tilquin, A. & Kokko, H. (2016). What does the geography of parthenogenesis teach us about sex?
769 *Philos. Trans. R. Soc. B Biol. Sci.*, 371, 20150538
770
771 81. Vulić, M., Dionisio, F., Taddei, F. & Radman, M. (1997). Molecular keys to speciation: DNA
772 polymorphism and the control of genetic exchange in enterobacteria. *Proc. Natl. Acad. Sci. U. S. A.*,
773 94, 9763–7
774
775 82. Watt, V.M., Ingles, C.J., Urdea, M.S. & Rutter, W.J. (1985). Homology requirements for
776 recombination in *Escherichia coli*. *Proc. Natl. Acad. Sci.*, 82, 4768–4772
777
778 83. Wilson, C.G. & Sherman, P.W. (2013). Spatial and temporal escape from fungal parasitism in
779 natural communities of anciently asexual bdelloid rotifers. *Proc. R. Soc. B*, 280, 20131255
780
781 84. Zawadzki, P., Roberts, M.S. & Cohan, F.M. (1995). The log-linear relationship between sexual
782 isolation and sequence divergence in *Bacillus* transformation is robust. *Genetics*, 140, 917–932

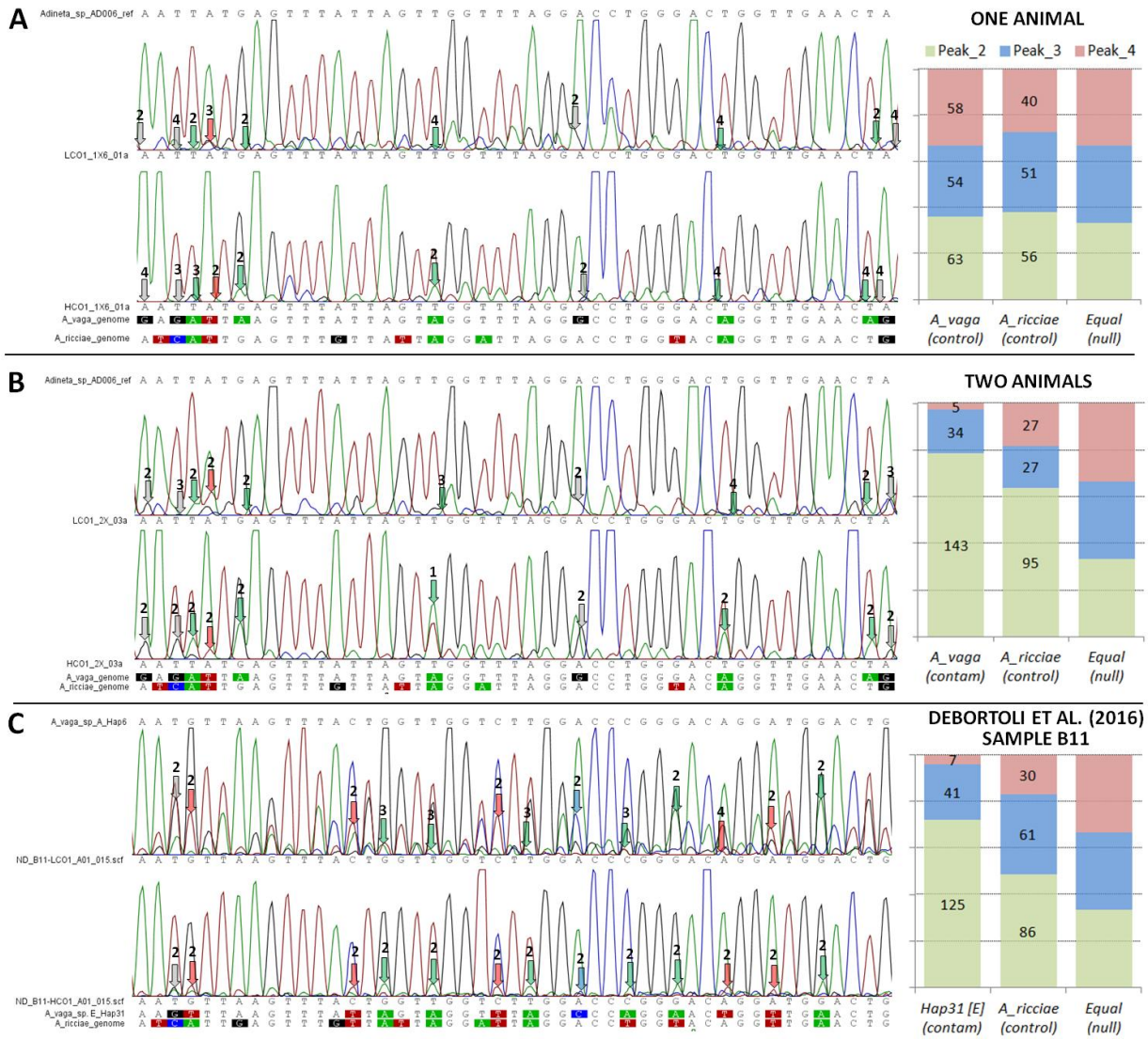

Figure 1. Contingency table analysis of minority peak height ranks (ConTAMPR), illustrated for mtCO1 chromatograms. **A.** For a sample with a single animal, the height ranks of minority peaks matching other rotifer sequences did not differ significantly from an equal distribution (χ^2 = 3.32, d.f. = 4, P = 0.51). **B.** When two animals were deliberately added to a sample tube, the minority peaks were a significantly better fit to the known sequence of the second animal (*Adineta vaga*) than either the null expectation (χ^2=88.15, d.f. =2, P <2.2 x 10^{-16}), or a control rotifer sequence (*A. ricciae*; 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 significantly better fit to the predicted sequence of a suspected second animal (*A. vaga* Species E 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, d.f. =, P = 3.07 x 10^{-6}). Minority peaks matching the focal query sequence are pointed out for a short illustrative region; numbers indicate peak height ranks. Bar graphs show the total peak height rank distributions for each sequence across the full length of the aligned locus (~605bp), and the null expectation if the peaks represent random noise.

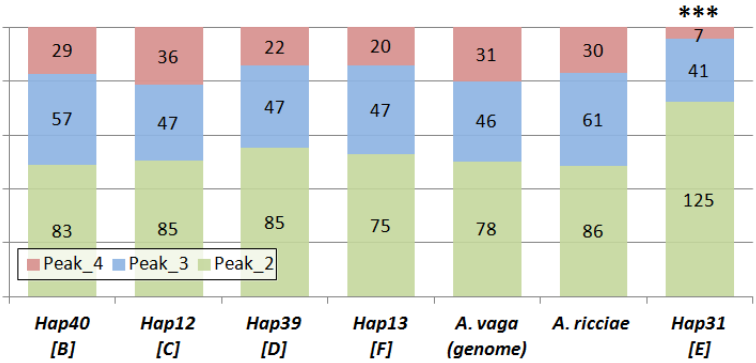

Figure 2. The mtCO1 chromatograms of Debortoli et al. (2016) for Sample B11 ("Individual 21" [A]) indicate a second animal belonging to Species E. The minority peaks fit Hap31[E] significantly better than six control sequences (***: χ^2=39.2, d.f. =12, P = 9.73 x 10^{-5}), which represent two reference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

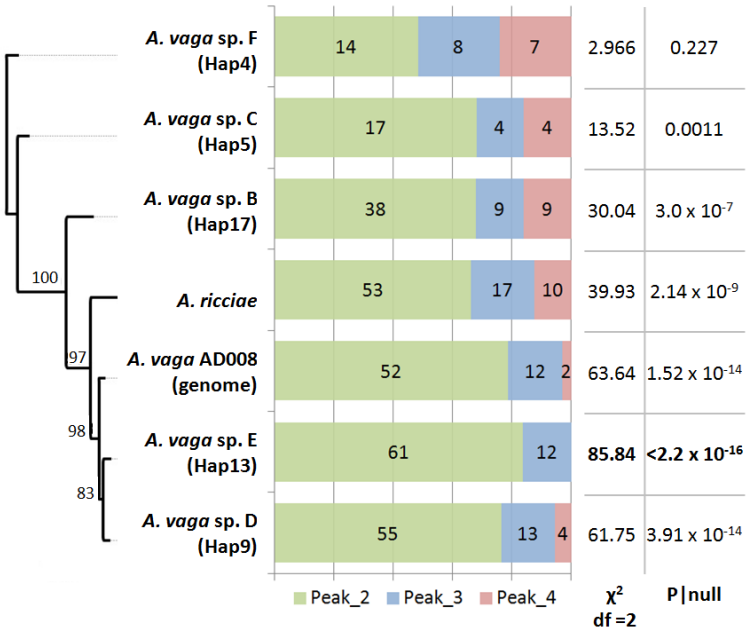

Figure 3. Minority peaks in 28S chromatograms for Sample B11 of Debortoli et al. (2016) indicate a second animal belonging to Species E. The probability of obtaining a peak rank distribution this extreme given random noise is lowest for Species E and increases for more distantly related control species,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

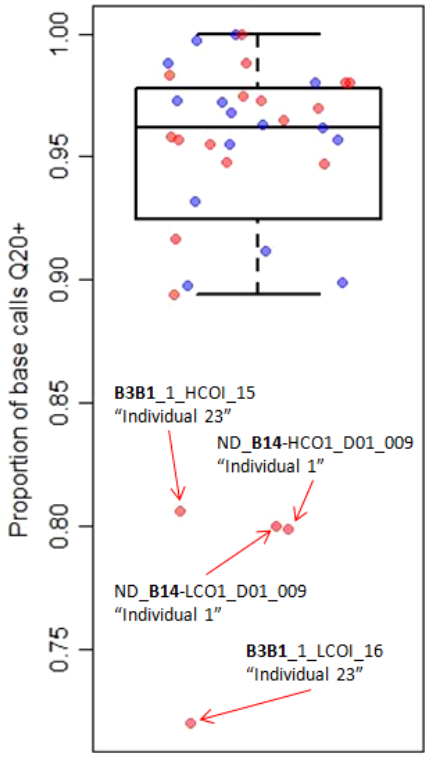

Figure 4. Phred quality scores for the subset of mtCO1 chromatograms provided by Debortoli et al. Red points represent samples where interspecific recombination was claimed; blue points represent samples where no such claim was made. The two groups do not have significantly different distributions (Mann-Whitney 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 sequences from three different species (Table 1). We suggest DNA from three animals was present.

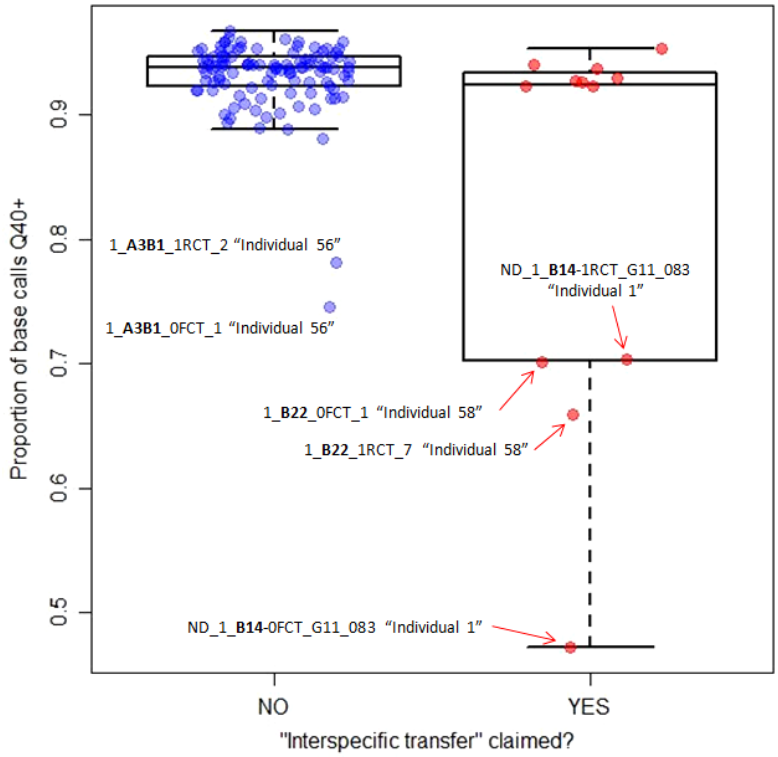

Figure 5. Phred quality scores for 28S chromatograms. Samples where Debortoli et al. reported "interspecific horizontal genetic transfers" have a significantly lower distribution (Mann-Whitney Test: N=122, W=373.5, P=0.015) because multiple 28S sequences are more often evident as minority peaks. This indicates that DNA from multiple animals was present in these samples, and perhaps also in some samples where "transfer" was not reported (e.g. A3B1). Sample B14 gives an extreme datapoint with a very prominent second sequence, but the two groups still differ significantly even if this is removed from the analysis (Mann-Whitney Test: N=121, W=373.5, P=0.04).

Sample code	Interpreted as	Sequences reported by Debortoli et al. (2016)					Additional sequences indicated by ConTAMPR	As seen also in	P (fit) given random noise P (fit) versus {control sequences}
		mtCO1	28S	EPIC25	EPIC63	Nu1054			
B11	Ind 21 [A]	Hap6 [A]	Hap1 [A]	Hap35 [E]	Hap16 [E]	Hap22 [E]	**mtCO1 c.f. Hap31 [E]**	Ind 81 [E]	$<2.2 \times 10^{-16}$ 9.73×10^{-5} {B;C;D;F;Ar;Av}
							28S c.f. Hap16 [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 c.f. Hap10 [C]**	Ind 50 [C]	9.1×10^{-4} 0.187 {B;D;E;F;Ar;Av} n.s.
							28S c.f. Hap16 [E]	Ind 81 [E]	$<2.2 \times 10^{-16}$ 3.0×10^{-4} {B;C;F;Ar}
							EPIC25 c.f. Hap30 [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 c.f. Hap36 [E]**	Ind 80 [E]	$<2.2 \times 10^{-16}$ 3.99×10^{-10} {A;B;D;F;Ar;Av}
							28S c.f. Hap16 [E]	Ind 72 [E]	3.69×10^{-10} 0.029 {A&B&F}
							EPIC25 ≠ Hap37 [E]	Ind 72 [E]	NA (>7 self-evident polymorphisms)
B3B1*	Ind 23 [A]	Hap1 [A]	Hap1-Hap2 [A]	Hap35 [E]	Hap1 [A]	Missing	**mtCO1 c.f. Hap11 [C]**	Ind 33 [C]	0.00158 0.99 {B;C;D;E;F;Ar;Av} n.s.
							EPIC25 c.f. Hap10 [C] (& c.f. Hap6 [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 c.f. Hap31 [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. Summary of six samples with incongruent species assignments among marker loci, which Debortoli et al. (2016) 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 sequences closely comparable to those seen in other animals in the dataset. These either matched the putative donor species, or the 'native' sequence that was supposed to have been replaced, indicating that incongruence was caused by cross-contamination. The height rank distributions of minority peaks corresponding to additional sequences 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 sequences, and calculated the probability that the fit of the focal sequence shared the same distribution (final column, lower values). "Ar": *A. ricciae*; "Av": *A. vaga* (reference genome). Asterisks indicate samples where sequences from three different species were recovered from the same sample.

Sample	Interpreted as	Marker	Claimed replacement of DNA 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)**

856 **Table 2.** Pairwise identity between DNA molecules that have undergone very recent "interspecific
857 genetic exchange", according to Debortoli et al. (2016). This degree of sequence divergence is not
858 compatible with current understanding of homologous recombination, or with the tetraploid
859 structure of the *A. vaga* genome. However, it is consistent with the alternative hypothesis of cross-
860 contamination. Divergences typically reflect substantial GC content differences among species.