SUPPLEMENTARY MATERIAL

1.0 Experimental cross-contamination: supplementary methods and results

1.1 Rotifer clones

To test the effect of adding two different animals to one tube, we selected two bdelloid rotifer clones from our cultures. One is the clone that provided DNA for the reference genome of *A. vaga* (Flot et al. 2013), which was kindly provided by K. Van Doninck in 2013. We believe it was collected originally in Italy (Mark Welch & Meselson 1998). We designate this clone "*A. vaga* (AD008)" or "*A. vaga* (genome)". The second clone was isolated from *Brachythecium rutabulum* (Hedwig), growing on *Quercus* sp. at Silwood Park, Ascot, UK (51° 24' 32.06" N 0° 38' 41.71" W), kept in continuous culture since 2012-01-09. We call this clone "*A.* sp. (AD006)". According to Debortoli et al. (2016), interspecific recombination occurred most often between the "cryptic species" A and E, and between C and E. These pairs of species share 86.1% and 86.4% sequence identity respectively at the mtCO1 marker. The identity between AD008 and AD006 at the mtCO1 marker is 86.5%, and thus commensurate with the species pairs involved in potential cross-contamination. Both clones were cultured using methods described previously (Wilson & Sherman 2010). Every two to three weeks, bdelloid populations were moved to fresh dishes of sterile distilled water over Czapek-Dox 0% agar (Barron 2004). Cultures were fed with a standardised inoculum of *Escherichia coli* (OP50), and *Saccharomyces cerevisiae* (S288c).

1.2 Design and replication

We prepared 12 tubes, divided into three groups and replicated as shown in Table S1. Biological replicates refer to different tubes, technical replicates refer to repeated PCR and sequencing using the same tube of template DNA. For each of the groups (1X6, 1X8, 2X6-8), one biological replicate was selected for technical triplication. For the 2X6-8 group, a further two biological replicates were selected for technical duplication.

1.3 Rotifer isolation

For our experiment, it was critical to be certain of the exact number of rotifers in each tube. The methods and citations provided by Debortoli et al. (2016) do not describe the technique used to isolate animals. We requested a protocol from the authors, and were given the following summary:

"Our procedure is simple, we collect the lichen/grass patch and put it in Spa® water overnight. The next day, we isolate the individuals identified as *A. vaga* by pipetting and washing them in clean water drops (serial dilutions). We then carefully checked under the binocular each tube to make sure that only one individual was present." (N. Debortoli and K. Van Doninck, pers. comm.)

This description raised some technical concerns for us. Prior to 2014, we had employed a similar procedure, but we found that pipettes were unsuitable for systematic isolation of individuals from nature, and that quality control procedures requiring visual inspection of Eppendorf tubes were inherently unreliable. Bdelloid rotifers are tiny, transparent and often rest motionless and invisible under the distortion of a meniscus or against the plastic base of a tube. They frequently stick inside pipette tips and are very difficult to dislodge or even to see. This leads to lost time and plasticware, and more seriously, to loss of specimens and bias in the subset of animals that successfully pass through the protocol. We occasionally experienced contamination via the following events. An individual is serially washed and deposited into a tube by pipette. The tube is carefully checked under the binocular microscope, but no animal is detected. It is assumed that the specimen was stuck in the pipette tip, but this cannot be directly verified. The tip is changed, and a second rotifer is serially washed and placed in the tube. This time, when the tube is carefully checked, a rotifer is visually confirmed, and the tube is sealed for DNA extraction. In fact, the first animal also entered the tube, but was hidden under the meniscus or on the bottom; thus, two rotifers now share one tube. This is not a particular problem when isolating animals from a clonal culture, but becomes a critical technical issue when isolating genetically different animals from nature. Opportunities for contamination increase when large numbers of animals must be isolated.

To address these problems, we now use a needle-based protocol when isolating individuals. This protocol is described in Additional File 1, and was used to prepare experimentally cross-contaminated samples. Into each of the 12 experimental tubes, 8µL of sterile Milli-Q water was pipetted. To each tube in groups 1X6 and 2X6-8, the needle protocol was used to move a single rotifer from a stock culture of *A. sp.* (AD006), via a wash droplet of 1mL sterile Milli-Q water. To each tube in groups 1X8 and 2X6-8, a single rotifer was moved from a stock culture belonging to *A. vaga* (AD008). The tubes in group 2X6-8 therefore contained two rotifers, one from each species.

1.4 DNA extraction and sequencing

We extracted DNA from the samples and amplified the mitochondrial cytochrome oxidase I (mtCO1) marker by PCR using the methods described by Debortoli et al. (2016). We used the same primers (LCO1 and HCO1; Folmer et al. 1994) at the same concentrations, and the same concentration of template in the same reaction volume (25μL). Amplifications were performed using GE Healthcare illustraTM PuReTaq Ready-To-Go PCR Beads. PCR products were purified, and sequenced in both directions with the same primers using an ABI 3730xl DNA Analyzer (Applied Biosystems), via a commercial Sanger sequencing service (Macrogen Europe, Amsterdam, The Netherlands).

1.5 Replicability of results

Technical replicates within the 2X6-8 group were concordant and the same clone always dominated the amplicon pool, but biological replicates showed different dominant clones (Table S2). For three samples, *A. vaga* (AD008) supplied the majority haplotype (99.5%, 99.3% and 99.6% of bases called); for the other three samples, *A. sp*. (AD006) was in the majority (99.6%, 99.7%, 97.9%). Concordance among technical replicates suggests that small differences in efficiency of lysis or DNA extraction are at least as important as differences during PCR in determining which of the genomes is amplified. Consequently, it may not be surprising to see a consistent majority sequence when amplifying repeatedly from the same sample, even if it contained multiple animals. The discordance among biological replicates suggests that the direction of the bias may be inconsistent between samples, even when the same two species are involved.

Figure 1 presented results from bidirectional pairs of sequencing chromatograms for Samples 1X6_01a and 2X6-8_03a, where *A. sp*. (AD006) was in the majority and *A. vaga* (AD008) in the minority. To check robustness in the reciprocal case, we repeated the analysis for Samples 1X8_02c and 2X6-8-05a, where *A. vaga* (AD008) was in the majority. The outcomes were as expected (Figure S1). Again, the minority peaks for AD008 often were hidden within noise associated with polymerase slippage and other errors, but the contaminant was recovered via ConTAMPR.

2.0 Methods for chromatogram quality analysis

Sequencing chromatograms for our experimental samples were returned by Macrogen Europe in the ABIF format, which included phred quality scores (Ewing & Green 1998). Chromatogram files were provided by Debortoli et al. in the .scf file format. We used CodonCode Aligner (v. 7.0.1, CodonCode Corporation) to assign phred quality scores to these chromatograms, which were imported for further processing in Geneious (v. 8.1.9, Biomatters Ltd, Auckland, New Zealand; Kearse et al. 2012).

Chromatograms for mtCO1 were uniformly trimmed to 605bp to avoid sequencing artefacts near the priming sites, and phred Q20 quality scores are reported for this section. The boxplots shown in Figures 4 and 5 were produced using R (v. 3.3.1, R Core Team) with the default setting for whisker length, and annotated manually. The two distributions were plotted separately in Figure 4 because they were statistically different. Owing to obvious outliers, distributions of quality scores for the chromatograms of Debortoli et al. (2016) were not assumed to be normal, and were compared using the Mann-Whitney test, implemented in R through the "wilcox.test" function. The equivalent distributions for our new data were approximately normal, but the difference between groups was not significant whether analysed using a parametric (N=38, t=1.13, P = 0.26) or nonparametric approach (N=38, Mann-Whitney W=234, P=0.1186).

The 28S ribosomal marker was amplified by Debortoli et al. in four overlapping fragments. This locus is highly conserved among even distantly related species. We restricted all our analyses to the first fragment, amplified with the primers 28S0FCT and 28S1RCT (Debortoli et al. 2016). Among the six *Adineta* species reported by Debortoli et al (2016), this fragment has 70 variable sites in 700bp (10%), whereas only 24 variable sites are found in the remaining 1610bp (1.5%). We trimmed chromatograms to a uniform length of 659bp (28S0FCT) or 666bp (28S1RCT) and calculated phred quality scores for this informative region. We focused on the Q40 phred score because the overall quality of 28S chromatograms was higher than for mtCO1; we suggest that the whole-genome amplification step tended to increase the representation of a single template and reduced or eliminated competing signals, especially after further PCR.

The distribution of 28S quality scores was shifted significantly lower for samples where HGT was claimed (Figure 4, Mann-Whitney Test: N=122, W=373.5, P=0.015). Both the median and second-highest quartile of the "HGT" samples fall within the second-lowest quartile of the "non-HGT" samples. The HGT group also had significantly more files with scores below 80% (Fisher's Exact Test, 4 in 12 versus 2 in 121, P=0.0006**;** or if we treat paired chromatograms from the same sample as non-independent, 2 in 6 versus 1 in 60, P=0.019).

No attempt was made to assess quality scores for EPIC25 chromatograms, because even if DNA all comes from a single animal, these amplicons represent a pair of homologous intronic regions that frequently are separated by at least one insertion-deletion polymorphism (indel), which means two sets of peaks are superimposed out-of-phase in some regions (Debortoli et al. 2016), rendering quality scores uninformative.

3.0 Contingency table analysis of minority peak ranks (ConTAMPR)

3.1 Multiple sequence alignments

The sequences of all haplotypes delimited by Debortoli et al. (2016) were retrieved from GenBank (KU860573–KU861170), along with relevant sequences for the *A. vaga* reference clone (GQ398061; JX184001), *A. ricciae* (EF173187; KM043216) and *A. sp*. AD006 (KM043183). Multiple alignment of chromatograms and candidate sequences was performed using MAFFT v. 7.017 (Katoh et al. 2009), implemented in Geneious using the MAFFT plugin (v. 1.3.3). For some sequences, particularly mtCO1, this algorithm alone was sufficient to bring peaks for the majority and putative minority sequences into alignment with the reference haplotypes. This is because there are no indels between species for the mtCO1 marker, so peaks corresponding to amplicons from different templates are superimposed ('in-phase'), though we typically saw slight displacement of minority peaks by less than a base-width in one direction or another relative to the majority peak. This is illustrated in Additional File 2, using annotated screenshots from the visual interface of Geneious.

For 28S rDNA, a multiple alignment of all unique sequences for *A. vaga* Species A-F revealed three indels of 1-2bp within a single 100bp region of the focal first fragment (Figure S2). This occasionally created challenges in testing whether two sequences from different species were present in an amplicon population. For instance, Sample B14 was predicted to include haplotypes from Species A and E. There is a single 1bp indel between these species (at position 134 in Figure S2), which means the minority peaks in a forward chromatogram are predicted to run approximately in-phase with the majority peaks until the indel, then become misaligned by 1bp, whereas the minority peaks in a reverse chromatogram will show the opposite pattern. The contaminant might thus be mistaken for a polymerase slippage artefact (Mullis et al. 1994). To analyse such a pattern, it is necessary to manually shift the alignment of each chromatogram by 1bp around the indel, which means the majority peaks are out of phase with the aligned majority haplotype for part of its length. This

alignment shift is illustrated in Additional File 3. For clarity, we point out every base corresponding to the minority Species E haplotype in both directions. The pattern of multiple peaks changes exactly as predicted at the site of the 1bp indel between Species A and Species E, which thus represents further, sequence-independent evidence for the additional haplotype.

EPIC25 is a highly variable intronic marker with multiple indels of up to 12bp within and 23bp between *A. vaga* Species A-F. Superimposed sequences from different species are therefore displaced even further and more frequently than at 28S, which makes it challenging to align even one candidate haplotype to the minority peaks. Multiple manual adjustments to an initial MAFFT alignment were necessary to follow the minority haplotype after each indel. It would not be feasible to attempt to align haplotypes from more than two candidate species at once against these chromatograms, since the peaks and variable sites would almost never be predicted to coincide with both candidates. However, it was not necessary to align every species individually to test for the EPIC25 sequences listed in Table 1, because the predicted matches were so clear and the alignment constraints made a match to any alternative haplotype so improbable. Additional File 4 illustrates the alignment shifts necessary to follow a minority Species A haplotype running alongside a Species E haplotype with at least 5 dispersed indels of varying lengths. Each peak we interpret as corresponding to the minority haplotype is individually highlighted.

3.2 Peak rank assignments

After bidirectional pairs of chromatograms had been aligned with candidate sequences, we manually scored the relative heights of minority fluorescence peaks at each site where a candidate sequence differed from the majority haplotype. We examined the trace lines corresponding to the remaining three nucleotides. Where these formed clear peaks, we assigned ranks 2, 3 and 4 based on their relative heights, and recorded the rank corresponding to the required base for each candidate sequence. The absolute heights were not considered, because we determined experimentally that peaks corresponding to a known second animal may be very small, inconsistent or even absent (Figure 1). Where two peaks appeared equal in height, we increased the magnification of the chromatogram using the Geneious interface until a difference, however small, became clear. If no such difference was apparent, the available ranks were randomly assigned to the two peaks.

When the minority sequence was 1bp out of phase with the majority haplotype, minority bases sometimes did not correspond to distinct peaks, but produced either a trailing or a leading 'tail'

attached to the preceding or succeeding peak. In these cases, there was usually a local maximum or at least an inflection point which we took as the height of the peak. If a base did not correspond to a clear peak, local maximum or inflection point, we estimated the mean relative heights of the trace lines at that site, regardless of shape. Where the trace line for a nucleotide showed absolutely no signal at a site, we recorded rank "5" to indicate this feature, but treated it as a fourth-ranked peak call for the purpose of analysis. Very rarely, the trace lines for two nucleotides both were flat, with no fluorescence signal. In these cases, the two missing nucleotides were each annotated as "6", and these were later split equally between ranks 3 and 4 for analysis.

At a locus like mtCO1, where all sequences are 'in-phase', the majority peak by definition cannot be called to represent a minority variant. However, Additional Files 3 and 4 illustrate a complication that occurs when majority and minority peaks are not aligned in phase, as at 28S and EPIC25. Sometimes, the expected minority peak at a variable site will happen to match the out-of-phase majority peak. This is a pure coincidence, but it prevents an assessment of how high the predicted secondary peak would have been otherwise, and it is not trivial to predict how often this is expected to happen under the null hypothesis. Where the "minority" haplotype happened to match a shifted majority peak, we recorded "1" as the rank, and we took a conservative approach and simply excluded all of these first-ranked "minority" calls from contingency analyses.

3.3 Statistical analysis of contingency tables

If minority peaks are a consequence of noise, non-rotifer contaminants, polymerase slippage or other sequencing artefacts, then all else being equal we predicted that the peak ranks corresponding to any control rotifer sequence would not differ significantly from an equal distribution (i.e. a 1:1:1 ratio for second, third and fourth-ranked peaks). We validated this prediction using chromatograms from our "1X" experimental groups (e.g. Figure S1). Alternatively, if a second haplotype is present, the set of peaks corresponding to that haplotype ought to differ significantly from the null distribution, showing a significant bias in favor of second- rather than third-ranked peaks, and third- rather than fourth-ranked peaks. For each candidate haplotype, we tested whether the peak rank distribution differed significantly from the null hypothesis of a 1:1:1 ratio, using Pearson's Chi-squared test for count data (Agresti 2007), implemented in R via the "chisq.test" function.

In many cases, more than one alternative sequence produced a significant deviation from the null distribution. This is expected, because at many variable sites an alternative base is shared by more

than one rotifer species; therefore, the true matching sequence will also 'drag' the rank distribution of species that share bases. For example, in Figure 1B, the *A. ricciae* control sequence deviates from the null expectation (χ^2=64.9, d.f. =2, P = 8.07 x 10^{-15}), but only because it shares some variants with *A. sp*. (AD006). Excluding these shared sites abolishes the apparent fit (χ^2= 3.2, d.f. = 2, P=0.202). The effect of relatedness is further illustrated in Figure 3. To distinguish the primary match, we compared the degree of fit not only against the null distribution, but among different candidate species. We typically used the Chi-square test of independence for a 3 x n contingency table, where n is the number of candidate species or haplotypes. If the initial table included cell counts too small to meet the assumptions of the test, two or more control species were pooled. Table 1 indicates the species or haplotypes that were used for each comparison; distributions that were pooled are indicated with "&". In some cases, further pairwise contrasts are reported in the text. To correct for the problem of multiple comparisons, α was adjusted using the Bonferroni correction, but all hypothesis tests remained significant even using this highly conservative approach.

4.0 Neighbor-joining phylogeny

The phylogenetic tree in Figure 3 was constructed using the neighbor-joining method implemented in the Geneious Tree Builder tool, with the default settings and 100 bootstrap replicates.

5.0 Supplementary results for samples with evidence of "interspecific DNA transfers"

Analysis of chromatograms for the six apparently incongruent samples indicated that some contained DNA from more than one animal, while others contained the predicted "original copies" of genes that had supposedly been replaced via "interspecific horizontal genetic transfer". The results are summarised in Table 1, and this section discusses each of the samples in greater detail.

5.1 Sample B11

To test the hypothesis of cross-contamination for Sample B11, it was necessary to predict which 28S and mtCO1 haplotypes a putative second animal from Species E might have. We consulted Table S3 of Debortoli et al. (2016), and found that only Individual 81 [E] shares all the Species E haplotypes that feature incongruently in Sample B11. We therefore aligned the B11 chromatograms to the haplotypes of that individual: Hap31 [E] at mtCO1, and Hap13 [E] at 28S. The choice of control haplotypes to represent each of the other species (Figure 2) was random. Peak rank distributions for

the six control species were statistically indistinguishable from each other (χ^2=6.66, d.f. = 10, P = 0.76), and only differed from the null expectation because each happened to share some bases with Hap31 [E]. For example, if we exclude polymorphisms *A. ricciae* shares with Hap31 [E], it no longer differs from an equal ratio of second, third and fourth peaks (30:40:26; χ^2=3.25, d.f. = 2, P = 0.197).

Although ConTAMPR revealed additional mtCO1 and 28S haplotypes for Sample B11 (Figure 2; Table 1; Additional File 2), the corresponding chromatograms are not obvious outliers in terms of phred quality scores (Figure 4; Figure 5). At 28S, for instance, both chromatograms for Sample B11 lie within the interquartile range for samples where no HGT is claimed. This is consistent with the results of our experiments: even when multiple animals are present, we do not necessarily see obvious differences in chromatogram quality, since minority peaks may be very small, or absent.

If Sample B11 contained two animals, it is interesting that the majority haplotypes for mtCO1 and 28S were from Species A, whereas those at EPIC25, EPIC63 and Nu1054 were from Species E. This may simply reflect the chance outcome of two consecutive nonlinear amplifications (WGA and PCR). However, the guanine-cytosine (GC) content of the Species A haplotypes expected at EPIC25, EPIC63 and Nu1054 is much higher than the corresponding Species E haplotypes that Debortoli et al. recovered (+17.7%, +13.6%, +21.2% respectively), whereas GC differences were much lower at mtCO1 and 28S (+1.9%). The whole-genome amplification kit used by Debortoli et al. has a known bias in favor of templates with lower GC content (Han et al. 2012), and multi-template PCR also is sensitive to this parameter (Polz & Cavanaugh 1998). Substantial differences in GC content may have helped to skew amplification of the competing haplotypes, effectively masking Species A at EPIC25, EPIC63 and Nu1054. At mtCO1 and 28S, GC was not substantially different, and the identities of both animals were recovered via ConTAMPR, though their representation was far from equal. Owing to effects like this, absence of evidence is not evidence of absence when considering amplicons from potentially contaminated samples. Even if two animals are present, one may be masked by the other at certain loci or in certain amplifications. It is very difficult to exclude the hypothesis of contamination on the basis of apparently clean chromatograms, but it is immediately telling to discover an extra haplotype.

5.2 Sample B22

The predicted donor of the incongruent EPIC25 haplotype in "Individual 58" was Species E. In animals of this species, Table S3 of Debortoli et al. (2016) shows that EPIC25 Hap37 [E] occurs with

mtCO1 Hap29 [E] and 28S Hap16 [E]. For ConTAMPR, we therefore aligned the B22 mtCO1 and 28S chromatograms against these candidates, along with control sequences from other species. Again, we found evidence for the predicted haplotypes (Figure S3, Figure S4). The extra 28S haplotype in Sample B22 was sufficiently prominent that the phred quality scores for these chromatograms fell outside the range of values for samples where no HGT was claimed (Figure 4).

We examined chromatograms for Sample B22 at the EPIC25 marker itself, where the authors reported a single, incongruent haplotype: Hap37 [E]. We did not find evidence for an expected 'native' Species C sequence. We suggest this haplotype was lost either during PCR or WGA, perhaps in part because its GC content would have been approximately 22% higher than Hap37 [E].

The authors characterised "Individual 58" as "homozygous" at EPIC25. However, it is clear from the data that a second Species E haplotype also was present in Sample B22. The trace files show hundreds of double and triple peaks of comparable heights. As discussed above, these represent two genomic homologs, running slightly out of phase following an indel, and with triple peaks indicating further single nucleotide polymorphisms (SNPs) between them. There are at least 7 SNPs between Hap37 [E] and the other haplotype in Sample B22, along with the indels. Many of these appear to correspond to standing polymorphisms shared by other animals in the Species E population (Figure S5). The presence of two rather divergent "transferred" haplotypes in a putative "recipient individual" is important. It is not consistent with the HGT scenario posited by Debortoli et al. (2016), in which "interspecific recombination" replaced the original DNA at one site, and "gene conversion promptly copied the integrated DNA on its homologous region". That would produce two identical haplotypes in the recipient. It would not preserve various SNPs and indels found in a "heterozygous" donor. On the other hand, this pattern is predicted if the haplotypes arose from a contaminating animal belonging to Species E, which had heterozygous combinations at EPIC25 similar to those seen in Individuals 81 (Hprim14a/b) and 78 (B39a/b). This is an independent line of evidence for the conclusions supported by ConTAMPR at 28S and mtCO1.

5.3 Sample B39

Debortoli et al. (2016) interpreted Sample B39 as Individual 66 [E]. A Species E mtCO1 haplotype had been replaced, in their view, by one imported from Species C. An alternative hypothesis is that Sample B39 was cross-contaminated with some DNA from Species C, which happened to be amplified by the mtCO1 primers instead of the native sequence. This hypothesis predicts a Species E

haplotype congruent with the other loci among the minority mtCO1 amplicons. Specifically, Table S3 of Debortoli et al. (2016) indicates that Individual 66 ought to have mtCO1 Hap31 [E], as seen in Individual 81. Like Individual 66, that animal had Hap16 [E] at 28S, Hap16 [E] at EPIC63, Hap19 [E] at Nu1054 and Hap30 [E] at EPIC25. No other individual had such a combination.

We used ConTAMPR to test whether minority peaks might correspond to Species E rather than any other species, and also to Hap31 [E] rather than any other Species E mtCO1 haplotype. We aligned B39 chromatograms not only to other *Adineta* species, but to seven diverse haplotypes from Species E. We found strong and specific evidence for the 'missing' native mtCO1 haplotype predicted for Individual 66 (Figure S6). This result brings all five loci into concordance without the need to invoke "interspecific horizontal genetic transfer" or transformation of mtDNA, which is problematic in itself (Larosa & Remacle 2013). The only incongruence in Sample B39 is the presence of mtCO1 Hap 10 [C] among the amplicons. We attribute this either to chance amplification from loose Species C mtDNA associated with the surface or gut of Individual 66, or to a second animal whose nuclear sequences were dropped or outcompeted during WGA or PCR, as seen with samples B11 and B22.

5.4 Samples B14 and B3B1

For samples B14 and B3B1, the minority peaks in mtCO1 chromatograms were a significantly better match to several other bdelloid mtCO1 haplotypes than the null expectation. This is consistent with the presence of additional mtCO1 sequences, and therefore DNA from additional animals in these samples (Figure S7). However, the peak rank distributions for several candidate species could not be distinguished statistically when compared with each other.

Looking at other loci, we noticed that B14 and B3B1 were the only samples to show unambiguous evidence of haplotypes originating from at least three different species (in each case, A, C and E; Table 1). We guessed that we were unable to identify a single consistent secondary sequence at mtCO1 because three animals from quite different species had contributed DNA to these samples. This hypothesis was supported by the fact that mtCO1 chromatogram from these two samples were extreme outliers in quality (Figure 5). The presence of DNA from three animals would explain why additional mtCO1 haplotypes cannot be narrowed down to a single candidate. Relative to the subtle minority peaks produced when we added just one extra animal (Figure 1B), the noise in these chromatograms is such that almost any *Adineta* haplotype could be present. The hypothesis of

"interspecific horizontal genetic transfers" supplies no obvious explanation for the unusual features of the mtCO1 amplicon population from these particular samples.

For Sample B14, we found evidence of an additional haplotype at 28S, consistent with Species E and a better match by a significant margin than more distantly related species (Figure S8). This suggests that one of the additional animals belonged to Species E, which would explain the incongruent Species E haplotypes Debortoli et al. (2016) found at EPIC63 (Table 1). Peaks corresponding to this additional haplotype also explain the unusually low phred quality scores for the 28S chromatograms for Sample B14 (Figure 4). These peaks are annotated fully in Additional File 3. The absolute heights of secondary peaks were often very low, as we saw at mtCO1 when we deliberately added two animals. At 28S, this may reflect preferential nonlinear amplification of one haplotype during both WGA and PCR. This would explain why some of the other contaminated samples (e.g. B11) did not show obviously anomalous 28S quality scores, or sometimes any detectable second 28S haplotype (e.g. B3B1). Conversely, no horizontal exchange was claimed for Sample A3B1 ("Individual 56" [C]), yet it was another clear outlier in Figure 4. We were not provided with mtCO1 or EPIC25 chromatograms for Sample A3B1, but the evidence from 28S suggests that DNA from a second animal was present. Perhaps no interspecific recombination was claimed because the same animal's sequence happened to be in the majority at all loci. The true incidence of cross-contamination may therefore be higher than the six samples where incongruence was noted.

For Sample B14, we found evidence of an additional EPIC25 haplotype consistent with Species E, even though two EPIC25 haplotypes from Species C had already been reported by Debortoli et al. The match was better than the null expectation by a significant margin (χ^2=11.18, d.f. = 2, P = 0.00374). The presence of a third EPIC25 haplotype in a single sample is suggestive regardless of its identity, since no single animal in the study of Debortoli et al. had more than two copies of this marker. Only one chromatogram was provided (for the primer EPIC25F), and without the guidance of bidirectional reads for this indel-rich intronic marker, we did not attempt to align other species.

For Sample B3B1, the only EPIC25 haplotype reported by Debortoli et al. belonged to Species E. However, we found evidence of at least one and possibly two additional EPIC25 haplotypes uniquely matching Species C (Table 1). The fit was very significantly better than the null expectation (χ^2=108.44, d.f. = 2, P < 2.2 x 10^{-16}). Indeed, no other species could be aligned to the secondary peaks, given the indel issues discussed above. The model of "interspecific recombination" and "gene conversion" presented by Debortoli et al. (2016) has difficulty accommodating two different non-

native sequences at one locus, in addition to the other more serious obstacles we discuss elsewhere. The 'native' EPIC25 sequence we would predict for Species A was not recovered; we suggest it was dropped during WGA or PCR. At the noisy mtCO1 locus, Species C also showed the strongest evidence of a fit to the minority peaks (Figure S7, χ^2=12.9, d.f. = 2, P = 0.00158), though the fit to Species E also differed significantly from the null expectation (χ^2=7.35, d.f. = 2, P = 0.0254). This evidence points to Species C as one of the contaminants. Our interpretation is that Sample B3B1 contained animals or loose DNA belonging to Species A, C and E.

5.5 Sample D14

Debortoli et al. (2016) interpreted Sample D14 as "Individual 5", and inferred that a Species A EPIC25 sequence had been replaced by Hap10 from Species C. If the incongruent sequence instead reflects amplification from another animal or contaminating DNA from Species C, a native Species A haplotype is expected among the amplicons. Table S3 of Debortoli et al. (2016) seems to predict either Hap1 [A] or Hap4 [A]. In the chromatograms, Hap10 [C] is the majority sequence, but in both directions there is a second haplotype with peaks of almost equal height running several base pairs out of phase, as expected if indels are present. It corresponds exactly to Hap4 [A], with a specific number of GAA tandem repeats to distinguish it from Hap1 [A]. Additional File 4 shows these chromatograms aligned, pointing out peaks matching the second sequence, and highlighting sites where it differs from the Hap 10 [C] interpretation. It was not necessary to attempt to align all the other *Adineta* species to these chromatograms and compare peak heights, as EPIC25 is so variable that no other species would match. Almost all base calls for Hap4 [A] use either first- or second-ranked peaks, and no fourth-ranked peaks are required at all. The probability this could happen by chance is negligible (χ^2=75.97, d.f. = 2, P < 2.2 x 10^{-16}). The most parsimonious interpretation is that Individual 5 belonged to Species A, and had concordant haplotypes at all loci in the expected combinations. Sample D14 was contaminated with some DNA belonging to Species C, with an EPIC25 haplotype that was amplified by WGA and PCR along with the native haplotype.

As discussed in the text, Debortoli and colleagues supplied two separate bidirectional pairs of EPIC25 chromatograms for Sample D14. Each pair represented an independent PCR amplification from the same DNA sample following WGA (N. Debortoli, pers. comm.). In one chromatogram pair, discussed above, the expected Species A sequence was unambiguous. Importantly, however, this native sequence was absent from the second pair of chromatograms, even as minor secondary peaks (Additional File 4). Even on close scrutiny, only the incongruent sequence from Species C was visible.

Clearly, the DNA in the template tube had not changed from one PCR to the next; therefore, the inconsistency arose from complete loss of a haplotype during the dynamics of multi-template PCR.

6.0 Genetic identity and microhomology analyses

6.1 Pairwise marker alignments to determine homology between species

To determine pairwise homology between sequences involved in “interspecific recombination”, we aligned the putative donor and recipient haplotypes in each case as described above using the MAFFT v. 7.017 algorithm (Katoh et al. 2009), implemented via the Geneious plugin (v. 1.3.3) with default settings. We then recorded the pairwise identity for each alignment and the GC content of each sequence, as reported in the Geneious "Statistics" interface.

6.2 Validation of pairwise homology calculations for wider genomic regions

The genetic distances in Table 2 are estimated for short (<1kb) markers that Debortoli et al. (2016) selected for easy amplification. However, horizontal transfer events are claimed to extend several kilobases beyond the markers they encompass (Figure 5 of Debortoli et al. 2016). We considered the possibility that genomic regions beyond the focal markers might show substantially greater sequence homology between species, thereby decreasing the mechanistic implausibility of HGT. For example, the EPIC25 marker (ca. 400bp) spans the first intron of a gene approximately 4.3kb long, encoding a product with similarity to the vertebrate metastasis suppressor protein 1 (MTSS1). In the *A. vaga* reference genome (Flot et al. 2013), the next-closest gene is approximately 2kb from the marker in a 5' direction, and encodes a product with similarity to vertebrate trifunctional enzyme subunit beta (HADBH). Because introns are often highly variable, pairwise identity at the EPIC25 marker might underestimate the homology between two species for the shared genes in this region.

We tested this hypothesis for the "interspecific recombination" event inferred from Individual 58 (Sample B22), in which EPIC25 Hap10 [C] was putatively replaced by Hap37 [E]. At the marker itself, these haplotypes only shared 68.4% genetic identity. We wanted to determine whether the value might be higher if we considered the whole region. While investigating putative intraspecific exchange, Debortoli et al. (2016) sequenced a longer (10.8kb) region surrounding EPIC25 Hap10 for two other individuals in Species C (42 and 51). The region containing the genes *MTSS1* and *HADBH* was syntenic with the *A. vaga* reference genome. To determine the homology between Species C

and E, we would ideally align this extended region with its counterpart in Species E, but no sequenced genome for Species E is available. However, the reference genome clone of *A. vaga* ("AD008") happens to be very closely related to Species E (Figure 3). In fact, the percentage homology to Hap10 [C] at the EPIC25 marker is identical (68.4%) for Hap37 [E] and AD008. We compared the equivalent identities at all five available loci, and in each case, the homology to Species C was nearly identical for Species E and the *A. vaga* reference genome (Table S3). This coincidence enabled us to use the *A. vaga* reference genome as a surrogate for Species E, with some confidence that the results would reflect the relationship to Species C.

We aligned the sequenced EPIC25 region from Individual 42 [C] (GenBank: KU861136.1) against the matching region from the reference genome (Assembly GCA_000513175.1) using MAFFT as implemented in Geneious, with default settings. For clarity, we delineated a focal region from the stop codon of *MTSS1* to the stop codon of *HADBH* (8949bp, approximately centred on EPIC25). This included exons and introns from both complete genes, and the intergenic region. We then measured the pairwise identity between the species for variety of subregions (Table S4).

The homology between these species for the whole region is 62.3%, which is less than the estimate based on the EPIC25 marker. The intergenic region is very divergent (52.4%), but even if we only compare genes, the values are either lower than the estimate from the EPIC25 marker (for *HADBH*), or identical to it (for *MTSS1*). Thus, we can reject the hypothesis that the marker-based identities in Table 2 underestimate the homology between species for broader genomic regions. The distances involved would remain incompatible with interspecific recombination even if we only looked at discontiguous exons, whose identity was 75.1% for *MTSS1*. This is nearly the same as the mean identity between independently evolving ohnologs in the reference genome (74.8%, Flot et al. 2013).

6.3 Methods for microhomology analysis

Mechanisms of "interspecific recombination" based on overall homology seemed to be excluded for the sequence pairs we considered, which have equal or lower identities than independently evolving ohnologs within the same genomes (Table 2, Table S4). However, we also considered alternative mechanisms with less stringent identity requirements than HR (N. Debortoli, J.-F. Flot, K. Van Doninck, pers. comm). One example is homology-facilitated illegitimate recombination (HFID), in which "single regions of high nucleotide-sequence similarity (~200 bp in length)...initiate recombination events that lead to the additive integration of >1000-bp-long heterologous DNA

fragments" (Thomas & Nielsen 2005). Another is microhomology-mediated end joining (MMEJ), whose "foremost distinguishing property...is the use of 5–25 bp microhomologous sequences during the alignment of broken ends before joining" (McVey & Lee 2008). If interspecifically transferred sequences share longer or more frequent mismatch-free blocks than genomic ohnologs (Vulić et al. 1997), then HFID or MMEJ might explain how they could be exchanged frequently while ohnologs with higher global homology evolve independently.

We estimated the length and frequency of microhomologous blocks for genes surrounding the putative transfer inferred from Sample B22, between EPIC25 Hap10 [C] and EPIC25 Hap37 [E]. As above, we used the genome reference clone for *A. vaga* (AD008) as a surrogate for Species E (Table S3). At every scale from 1-40bp, we compared interspecific microhomology for *MTSS1-A* and *HADBH* with intragenomic microhomology for 7650 ohnologous pairs of genes in the *A. vaga* reference genome. As an important point of comparison, we highlighted the microhomology between *MTSS1-A* and its own genomic ohnolog (*MTSS1-B*), which contains the EPIC63 marker. Debortoli et al. (2016) implicitly assume there is no exchange between these highly divergent genes, which were treated as "independent nuclear markers" and sequenced with "ohnologue-specific" primers.

Gene models for *A. vaga* were constructed using BRAKER (Hoff et al. 2015), with RNASeq as evidence (SRA accession: ERR260376; Flot et al. 2013). Collinear regions were identified using MCScanX (Wang et al. 2012), setting the maximum number of allowed gaps between collinear genes to 10. Between all pairs of collinear genes, synonymous (K_S) and nonsynonymous (K_A) divergences were calculated using the method of Nei & Gojobori (1986), implemented in BioPerl (Stajich et al. 2002). Ohnologs were defined as pairs of genes within collinear regions with $K_S \geq 0.5$ (n = 7,650). Ohnologous regions (comprising exons plus introns) were extracted and aligned using MAFFT (Katoh & Standley 2013) with default settings. Introns were included because intronic markers were claimed to show interspecific recombination, and any inter-ohnolog recombination facilitated by microhomology would involve unspliced genomic DNA. Microhomology across all 7,650 alignments was calculated with a custom Perl script, using a sliding window approach along each alignment (sliding one base each iteration), from a window size of one to 40 bp, and counting any window of exact identity as a match. The number of identical blocks was scaled relative to the length of each alignment to account for variation in gene length, and multiplied by 1,000 to give a per-kb estimate. All scripts are available at https://github.com/reubwn/microhomology. The same method was used to measure microhomology between Species C and *A. vaga* for the alignments of *MTSS1-A* and *HADBH* discussed

above. Finally, we highlighted the specific microhomology curve in *A. vaga* corresponding to the pairing between *MTSS1-A* and its ohnolog, *MTSS1-B*.

Gene copies involved in putative interspecific recombination did not share significantly more or longer blocks of microhomology than independently evolving ohnologs in the same genomes, at any scale from one to 40bp (Figure S9). At most scales they even shared less microhomology with the "donor species" than with their own ohnologs. Microhomology-based mechanisms such as MMEJ and HFIR could not facilitate distant interspecific recombination and yet fail to permit exchange between ohnologs with even higher microhomology, especially as conspecific DNA fragments seem likely to be more abundant and available than heterospecific ones.

Other mechanisms of homology recognition seem even less applicable. For instance, pairing of chromosomes early in meiosis appears to be independent of recombination in some cases (Da Ines et al. 2014), but this involves chromosome-scale features such as centromeres and telomeres, which are not shared by loose DNA fragments. Pairing must still be stabilised by recombination, via sequence-dependent pathways. Another recognition mechanism involves homologous trinucleotide repeats interspersed at a specific periodicity within otherwise divergent sequences (Gladyshev & Kleckner 2016), but there is no evidence for this distinctive architecture in the putatively exchanged sequences we examined, and that pathway is not linked to recombination. It seems improbable *prima facie* that any mechanism could enable ready exchange of DNA across species boundaries, while simultaneously precluding exchange between less divergent ohnologs within the same genomes. We suggest that any argument to the contrary ought to bear the burden of proof.

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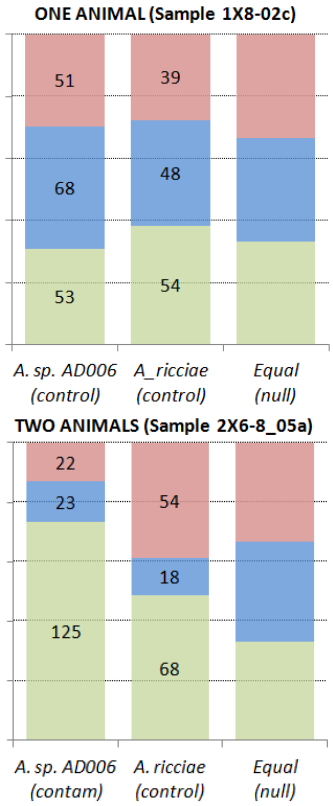


Figure S1. Minority peak analysis of mtCO1 chromatograms for two samples where *A. vaga* (AD008) was in the majority. In the first sample, *A. vaga* was the only rotifer present, and the fit of minority peaks to other haplotypes did not differ significantly from the null expectation. In the second sample, another rotifer belonging to *Adineta* sp. (AD006) was present, and minority peaks corresponding to this known contaminant were a significantly better fit than the null expectation (χ^2=123.61, d.f. = 2, P < 2.2 x 10^{-16}), or a control species, *A. ricciae* (χ^2=28.28, d.f. =2, P = 7.23 x 10^{-7}).

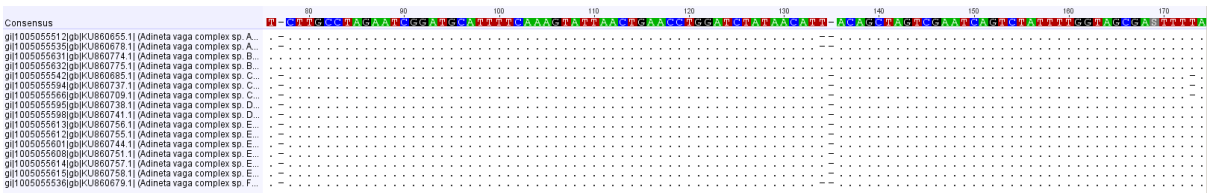
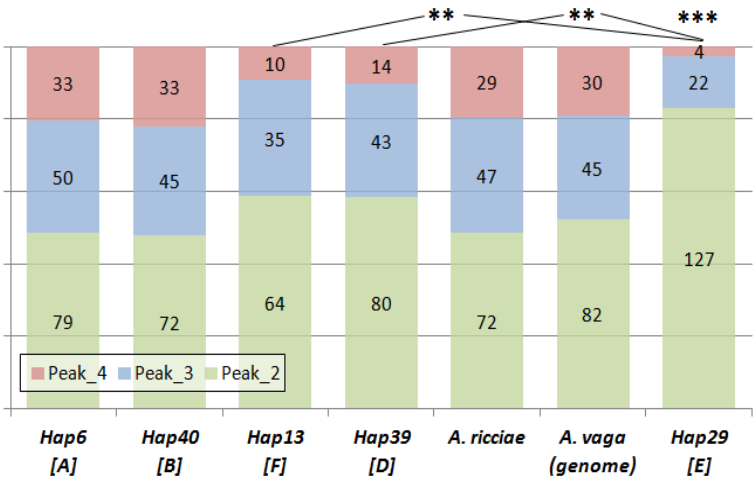


Figure S2. Insertion-deletion polymorphisms in an alignment of 28S ribosomal DNA sequences for the six *Adineta* species reported by Debortoli et al. (2016). Dots indicate agreement to the consensus sequence; dashes indicate gaps. Minority and majority sequences of different lengths are predicted to run out of phase in chromatograms for part of their length.



678 **Figure S3.** Summary of minority peaks in mtCO1 chromatograms for Sample B22. The fit of Hap29
679 [E] is significantly better (***) than the other six species (χ^2=69.49, d.f. = 12, P = 3.99 x 10^{-10}). If
680 Hap29 [E] is removed, the other distributions are not significantly different from one another
681 (χ^2=15.2, d.f. = 10, P = 0.124). If compared directly, Hap29 [E] is a significantly better fit (**) than
682 either Hap13 [F] (χ^2=19.48, d.f. = 2, P = 5.9 x 10^{-5}) or Hap 39 [D] (χ^2=22.2, d.f. = 2, P = 1.51 x 10^{-5}).
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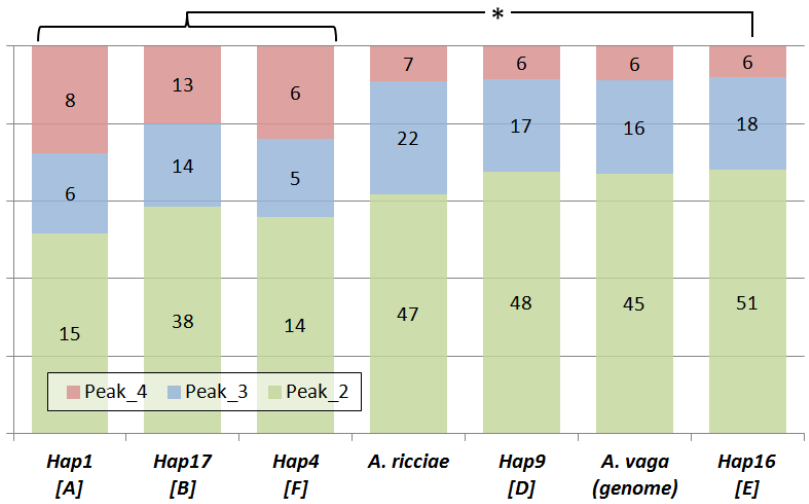

684 **Figure S4.** Summary of minority peaks in 28S chromatograms for Sample B22. Hap 16 [E] is a
685 significantly better fit to these peaks (*) than Hap1 [A], Hap 17 [B] and Hap4 [E], which were pooled
686 owing to small cell counts (χ^2=6.85, d.f. = 2, P = 0.029). The remaining species are too closely
687 related to Species E to be distinguished statistically at the highly conserved 28S locus.
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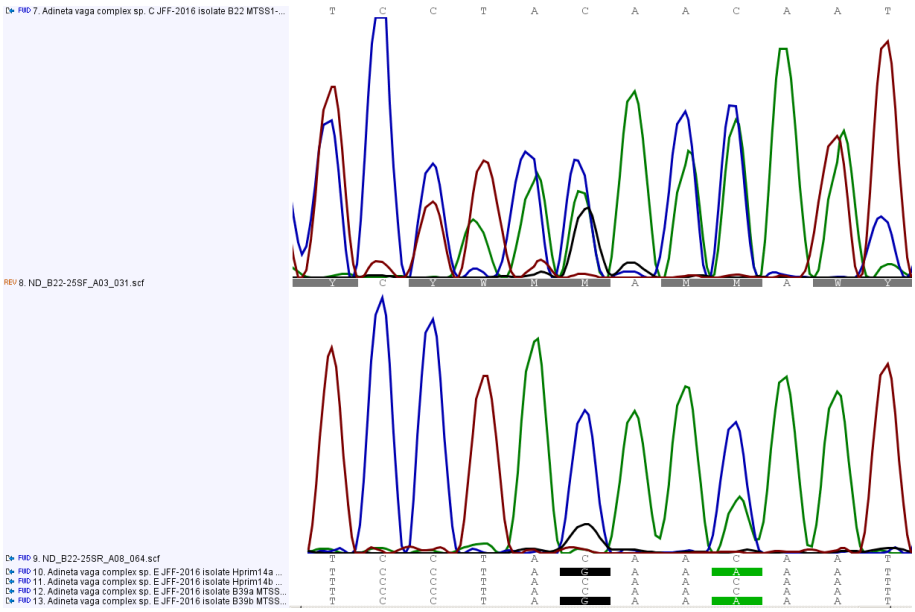

689 **Figure S5.** Shifted and double peaks in EPIC25 chromatograms for Sample B22 indicate indels and
690 SNPs between two homologous sequences, both belonging to Species E and incongruent with the
691 Species C background. The two SNPs shown here correspond to polymorphisms seen natively in
692 "heterozygous" animals from the Species E population (Hprim14a/b and B39a/b). Horizontal import
693 of standing heterozygosity is not predicted in the model of interspecific recombination presented by
694 Debortoli et al. (2016), but it is predicted if the sequences arise from cross-contamination.
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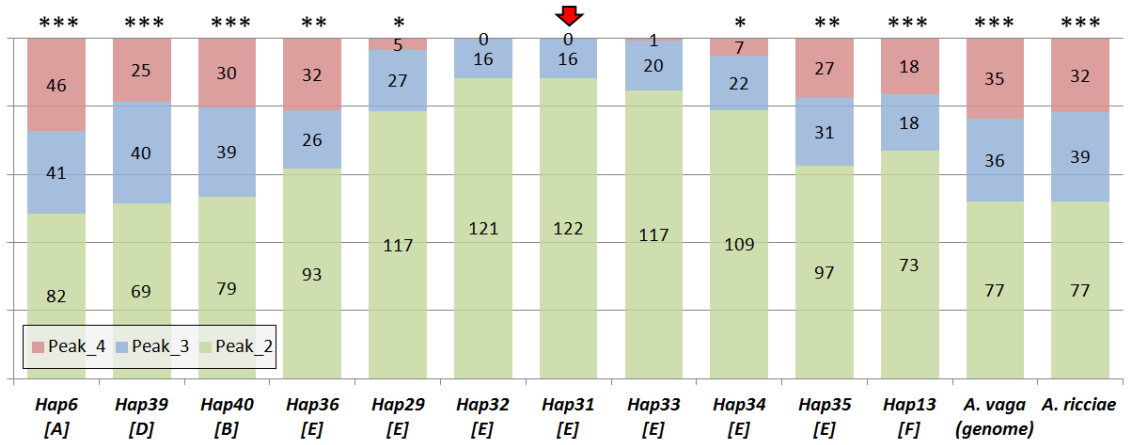

696 **Figure S6.** Minority peaks in mtCO1 chromatograms for Sample B39 (Individual 66) indicate the
697 predicted 'native' haplotype Hap31 [E] (red arrow), and challenge the hypothesis that mtDNA has
698 undergone interspecific recombination. Hap31 [E] is a significantly better match to the secondary
699 peaks than all other haplotypes (*: $\chi^2 = 9.63$, d.f. =4, P = 0.047; **: $\chi^2 = 66.71$, d.f. = 8, P = 2.21×10^{-11};
700 ***: $\chi^2 = 136$, d.f. = 20, P < 2.2×10^{-16}), except Hap32 [E] and Hap33 [E], which are nearly identical and
701 were not included in contingency table tests.

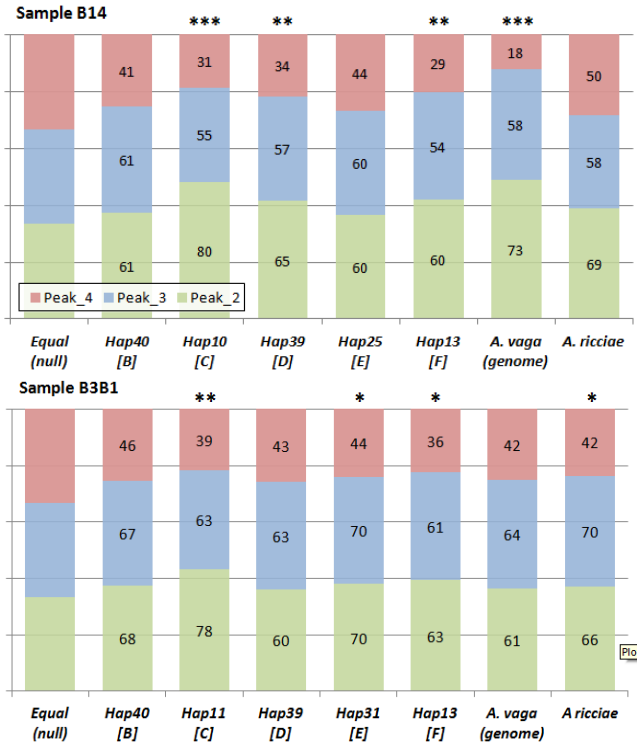

Figure S7. Analysis of minority peak distributions indicates additional mtCO1 sequences in chromatograms for Samples B14 and B3B1. Multiple candidate haplotypes are a significantly better fit than expected under a null distribution of peak ranks (*** : $P < 0.001$, **: $P < 0.01$; *: $P < 0.05$), but their distributions do not differ significantly when compared with each other, and we cannot therefore narrow down a single minority haplotype driving the pattern. Two different contaminating sequences may be superimposed in each case, alongside the Species A majority haplotype. Clear evidence for haplotypes from Species A, C and E was found at other loci.

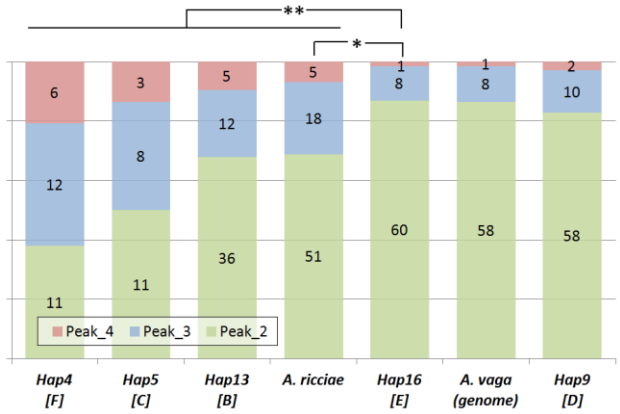

Figure S8. Minority peaks in 28S chromatograms for Sample B14 reveal a second haplotype that fits Species E significantly better than Species B, C, F or *A. ricciae* (**: $\chi^2 = 28.77$, d.f. = 8, $P = 3.0 \times 10^{-4}$; * : $\chi^2 = 7.1$, d.f. = 2, $P = 0.029$). Hap9 [D] and *A. vaga* (genome) are nearly identical to Hap16 [E] and cannot be distinguished statistically.

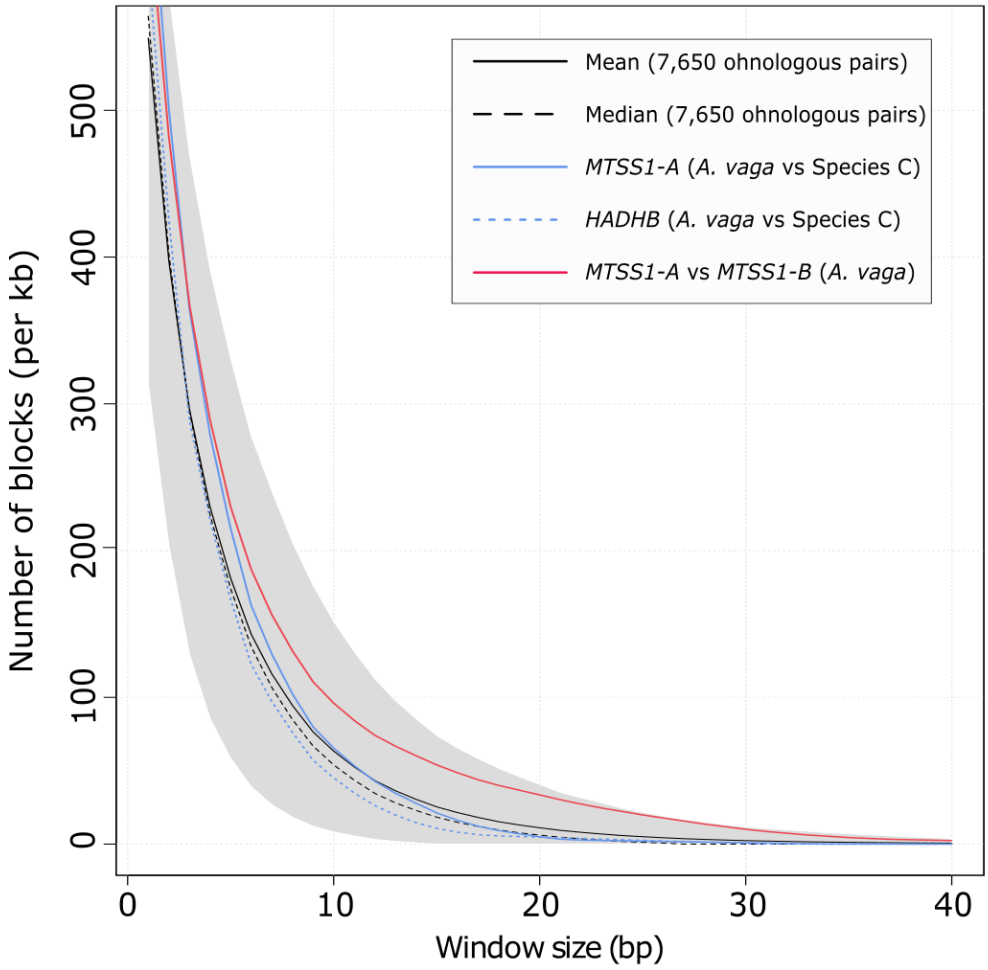

Figure S9. Two genes linked to an "interspecific horizontal genetic transfer" event did not share significantly more or longer microhomologous blocks than 7650 pairs of independently evolving ohnologous genes in the *A. vaga* reference genome. Regardless of scale (1-40bp), the degree of microhomology between "transferred" sequences (blue lines) falls within the 5% and 95% quantiles for genomic ohnologs (grey shading). The dashed blue line represents the gene *MTSS1-A*, containing the EPIC25 marker; the solid blue line shows the next-closest gene (*HADHB*). For sliding windows above 3bp, the "horizontally transferred" *MTSS1-A* sequences share less microhomology with each other than with their own independently evolving ohnolog (*MTSS1-B*) in the same genome (red line).

Group name	1X6	1X8	2X6-8
Animals	One: AD006	One: AD008	Two: AD006 and AD008
Biological replicates	3	2 (1 lost)	6
Technical replicates	1 triplicate, 2 single	1 triplicate, 1 single	1 triplicate, 2 duplicate, 3 single

Table S1. Design and replication of an experiment to determine the effect of multiple rotifers in a single DNA extraction tube. AD006: *A.* sp. 'AD006'; AD008: *A. vaga* (reference genome clone).

Sample code	Majority haplotype	Minority haplotype	Number of chromatogram files	Total bases called	Minority base calls	% sites with majority calls
01	*A.* sp. (AD006)	*A. vaga* (AD008)	2 (singleton)	1210	25	97.94
02	*A. vaga* (AD008)	*A.* sp. (AD006)	2 (singleton)	1210	5	99.59
03	*A.* sp. (AD006)	*A. vaga* (AD008)	4 (duplicated)	2420	8	99.66
04	*A. vaga* (AD008)	*A.* sp. (AD006)	2 (singleton)	1210	6	99.5
05	*A. vaga* (AD008)	*A.* sp. (AD006)	4 (duplicated)	2420	16	99.33
06	*A.* sp. (AD006)	*A. vaga* (AD008)	6 (triplicated)	3630	13	99.64

Table S2. Summary of base calls from ABI Sanger sequencing of mtCO1, corresponding to majority and minority haplotypes for biological and technical replicates of experimentally contaminated samples (Group 2X6-8). Chromatograms are always in bidirectional pairs.

Marker	Species C	Species E	C vs. E (%)	C vs. ref (%)
mtCO1	Hap10 (KU860596)	Hap29 (KU860588)	87.8	87.8
28S	Hap5 (KU860706)	Hap16 (KU860768)	97.6	97.7
EPIC25	Hap10 (KU860907)	Hap37 (KU860804)	68.4	68.4
EPIC63	Hap4 (KU860934)	Hap34 (KU860927)	67.4	67.6
Nu1054	Hap16 (KU861061)	Hap21 (KU861052)	70.1	70.2

Table S3. At five independent mitochondrial and nuclear marker loci, the percentage identity between Species C and Species E is almost exactly reproduced by comparing Species C to the reference genome of *A. vaga*. The reference genome therefore is an appropriate surrogate for Species E when estimating homology parameters for a hypothetical transfer from Species C.

Comparison	Aligned length (bp)	Identity (%)
EPIC25 marker only	395	68.4
Two-gene region	8949	62.3
MTSS1 whole gene	4419	68.4
MTSS1 exons	2301	75.1
MTSS1 introns	2118	61.1
Intergenic region	2203	52.4
HADBH whole gene	2327	60.1
HADBH exons	1605	67.0
HADBH introns	722	44.7

748

749 **Table S4**. Pairwise identity between Species C and the *A. vaga* reference genome for the region
750 surrounding the EPIC25 marker. Regardless of the scope or scale of the comparison, the distances
751 are not compatible with the "interspecific recombination" claimed between Species C and Species E.
752 The identity estimate from the short EPIC25 marker accurately reflects (or even overestimates)
753 homology more broadly.