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TECHNICAL COMMENT

EVOLUTIONARY BIOLOGY

Comment on "The hologenomic basis of speciation: Gut bacteria cause hybrid lethality in the genus *Nasonia*"

James Angus Chandler[1] and Michael Turelli[2]*

Brucker and Bordenstein (Reports, 9 August 2013, p. 667) claim that adaptive codivergence of gut bacteria with hosts contributes to hybrid lethality. Yet, they provide no evidence for coadaptation of bacteria and *Nasonia* hosts. Their data on hybrid viability suggest that bacteria contribute to inviability only because intrinsic hybrid dysfunction increases susceptibility to free-living bacteria. Hologenomic speciation remains testable speculation without experimental support.

Brucker and Bordenstein proposed that microbes contribute to speciation by concordantly diverging with their hosts, so that hosts become genetically incompatible with microbes found in sister lineages (*1*–*3*). They describe "hologenomic" speciation as an extension of the classic Dobzhansky-Muller (DM) model for hybrid inviability and sterility, based on the accumulation of incompatible alleles in evolutionarily distinct lineages (*4*–*6*). Their account (*1*) uses two novel phrases, hologenomic speciation and phylosymbiosis. We accept their definitions for both phenomena but reject their proposed evidence. Although their *Nasonia* data (*1*) convincingly demonstrate that bacteria contribute to hybrid lethality (specifically, germ-free rearing significantly increases F_2 male viability), they provide no evidence that lineage-specific incompatibilities between gut microbes and host genomes—essential to "hologenomic" speciation—contribute to this effect. We propose a simple alternative, termed "intrinsic hybrid dysfunction," in which DM incompatibilities between host genomes make hybrids more susceptible than parentals to any free-living bacteria—irrespective of the bacteria's recent history of association with the hosts. In addition to this central defect in their case for a novel speciation mechanism, Brucker and Bordenstein provide weak evidence for "phylosymbiosis," defined as concordant phylogenies between gut microbiota and hosts, and for "species-specific" microbial communities. We describe methodological flaws in their proposed evidence supporting these subsidiary claims.

How might hologenomic speciation be distinguished from intrinsic hybrid dysfunction? It is reasonable to suppose that as hosts diverge, they differentially acquire microbes from their environments (*7*). Hence, replacing the native gut community with microbes typical of other species might systematically lower host fitness [e.g., (*8*)], possibly by an amount that varies with the recipient's phylogenetic distance from the source host. Although (*1*) presents various results involving experimental inoculation with alternative microbes, there is no direct test of coadaptation by cross-inoculation of gut microbes between the three focal *Nasonia* species.

Without phylogenetically informed cross-inoculation experiments, data demonstrating that microbes can reduce hybrid viability are consistent with, but do not verify, hologenomic speciation. Moreover, the hybrid data in (*1*) seem more compatible with intrinsic hybrid dysfunction. In particular, Brucker and Bordenstein provide no examples in which the reduction of hybrid fitness can be associated with decreased levels of coadaptation between the hybrids and their gut bacteria. Instead, they show that (i) bacteria not derived from *Nasonia* [*Escherichia coli*; fig. S2A in (*1*); hybrids *g*/*v* and *v*/*g*; *g*, *N. giraulti*; *v*, *N. vitripennis*] and (ii) relatively rare bacteria from the parental *Nasonia* species [*Enterococcus*; fig. S1B in (*1*); purple; hybrids *g*/*v* and *v*/*g*] both reduce hybrid fitness by an amount indistinguishable from that seen in conventionally reared hybrids. Furthermore, providing hybrids with a microbiota that approximates that of both parents (i.e., predominantly *Providencia*) does not increase hybrid viability [fig. S1B in (*1*); teal; hybrids *g*/*v* and *v*/*g*], contrary to the expectation that more subtle disruption of the native microbiota should produce lower fitness costs. The experiments in (*1*) show that many bacteria, not just those found in the potentially abnormal bacterial communities of hybrids, increase hybrid lethality, as expected under the intrinsic-hybrid-dysfunction hypothesis. Without inoculation experiments that show a phylogenetic signal, the data on hybrid viability and data concerning expression levels for immune genes remain fully consistent with intrinsic hybrid dysfunction.

Brucker and Bordenstein suggest that additional support for hologenomic speciation is provided by phylosymbiosis (*1*). Phylosymbiosis, which is common in maternally transmitted symbionts, does not imply hologenomic speciation and is not required by it, but could facilitate it. Brucker and Bordenstein (*1*) used weighted (abundance based) and unweighted (presence-absence based) UniFrac, a phylogenetically informed distance metric (*9*), to support phylosymbiosis in their *Nasonia* clade. However, a reanalysis of their data using resampling to provide bootstrap support (*9*) finds weak support for concordant divergence (Fig. 1). Brucker and Bordenstein (*1*) present no statistical support values for their unrooted trees. Moreover, they previously (*3*) found no evidence for phylosymbiosis in *Nasonia* larvae, using either weighted or unweighted UniFrac [but did find weak evidence for phylosymbiosis in pupae and adults, life stages not analyzed in (*1*)]. Alternatives to UniFrac, such as nonphylogenetic abundance-based metrics, have been successfully used for evaluating host-microbiota congruence (*7*). However, when we use various alternative beta-diversity metrics [available in the software package Quantitative Insights Into Microbial Ecology (QIIME) (*10*)], we find no evidence that bacterial community structure recapitulates the host phylogeny (Fig. 2). Thus, while phylosymbiosis is possible in *Nasonia*, the evidence is much weaker than suggested in (*1*). Weak support for phylosymbiosis is hardly surprising, given that phylogenetic discordance is common in trophic associations not based on maternal transmission [e.g., (*11*, *12*)]. Indeed, phylogenetically discordant trophic associations may be more likely to facilitate speciation (*13*).

Another aspect of hologenomic speciation proposed for *Nasonia* are "species-specific" microbial communities (*1*)—i.e., communities structured by host genetic factors that produce differential microbial colonization or survival. Although certain operational taxonomic units (OTUs) are present within a single host species in (*1*), most species-specific OTUs are extremely rare (<1% of the total community). To determine whether these rare bacterial OTUs result from host-microbe interactions, rather than sampling artifacts, replicates from each host are needed, and different hosts should be co-reared in the same environment. No replicates are presented in (*1*), and although species specificity in *Nasonia* adults and pupae (but not larvae) was claimed in an earlier publication (*3*), those results were obtained by resampling the same long-established laboratory populations multiple times. Taken together, insufficient evidence supports the claim that the gut bacteria of *Nasonia* larvae are species specific.

Brucker and Bordenstein's data demonstrate that bacteria can contribute to hybrid lethality, but not because of concordant phylogenetic divergence with their hosts. The data suggest that hybrids may be generally weakened and incapable of dealing with many free-living bacteria. There are many such examples in both animals and plants (*2*). Intrinsic hybrid dysfunction is fully

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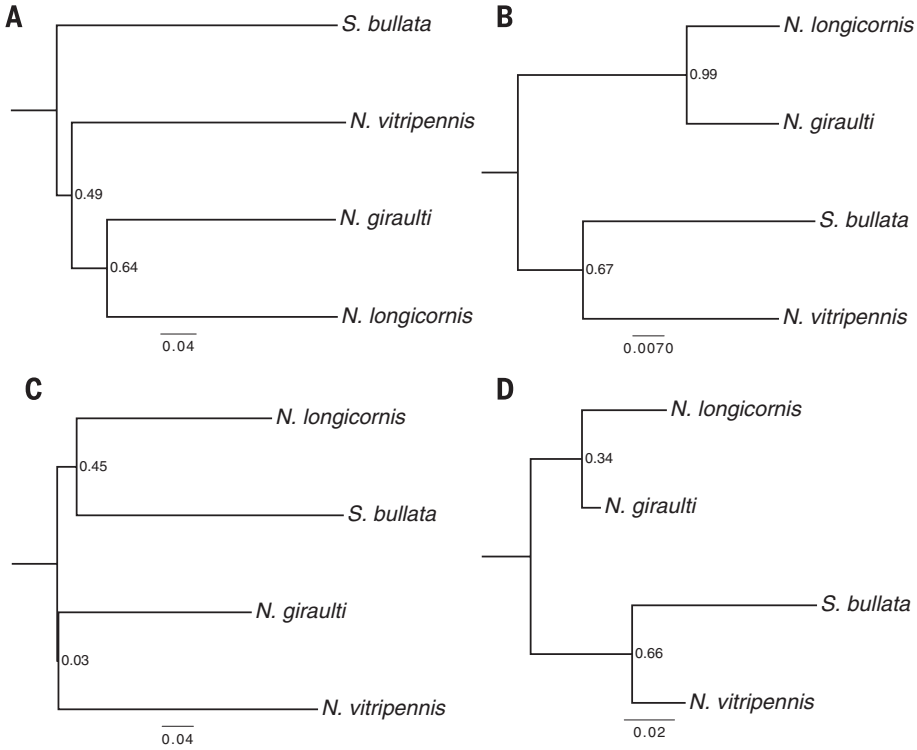

Fig. 1. Bootstrapped clustering using the UniFrac distance metric. Raw sequences were obtained from the Dryad Digital Repository (doi:10.5061/dryad.5r09q). The data were processed following the recommendations of the QIIME tutorial (http://qiime.org/tutorials/tutorial.html), except that OTUs were formed using the 95% similarity threshold [following the method described in the supplemental materials of (*1*)]. The details of our analyses are provided at http://figshare.com/articles/Hologenomic_speculation_on_Nasonia_speciation/1084395. Any sequences classified as *Wolbachia* or chloroplasts or that could not be classified as bacteria were removed from the data set. To obtain bootstrapped replicates, data were subsampled to 206 reads [75% of smallest library size; again as recommended by the QIIME/UniFrac developers (*9*) and http://qiime.org/tutorials/tutorial.html]. Support values represent the proportion of 100 subsampled trees supporting each node of the consensus topology. The clustering algorithms employed [unweighted pair group method with arithmetic mean (UPGMA) and neighbor joining] produce rooted trees; no outgroup is selected a priori. The results provide weak support for the "phylosymbiosis" prediction that the bacterial communities recapitulate the host's phylogeny: *Sarcophaga bullata* in not consistently identified as the outgroup with *N. girault* and *N. longicornis* as sister species. We used neighbor joining in addition to the QIIME default of UPGMA, because there is no reason to assume equal rates of divergence along different branches. (**A**) Unweighted UniFrac, UPGMA clustering. (**B**) Weighed UniFrac, UPGMA clustering. (**C**) Unweighted UniFrac, neighbor-joining clustering. (**D**) Weighted UniFrac, neighbor-joining clustering.

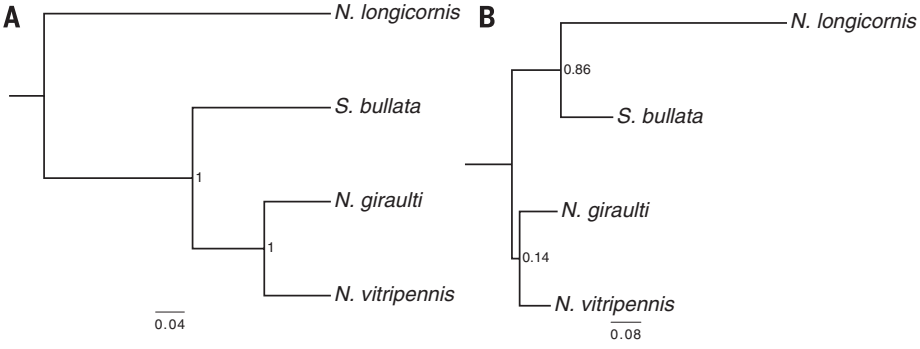

Fig. 2. Bootstrapped clustering using the Bray-Curtis distance metric. Data were processed as in Fig. 1, but an alternative metric was used to quantify bacterial community differences. Other abundance-based beta-diversity metrics (chi-square, chord, Euclidian, Hellinger, Kulczynski, Manhattan, Morisita-Horn, and Pearson) find the same topologies and similar support values (data not shown), again providing little support for phylosymbiosis. (**A**) UPGMA clustering. (**B**) Neighbor-joining clustering.

consistent with the standard DM model that host divergence leads to defective hybrids, without invoking coadaptation between hosts and their microbiota as a driver of speciation. Vertically transmitted microbes have been convincingly implicated in certain cases of speciation (*14*, *15*). However, the "hologenomic" conjecture that incompatibilities between lineage-specific, free-living, horizontally transmitted microbes contribute to speciation remains testable speculation without experimental support.

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