TECHNICAL RESPONSE

EVOLUTIONARY BIOLOGY

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

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Chandler and Turelli postulate that intrinsic hybrid dysfunction underscores hybrid lethality in *Nasonia*. Although it is a suitable conception for examining hybrid incompatibilities, their account of the evidence is factually inaccurate and leaves out the evolutionary process for why lethality became conditional on nuclear-microbe interactions. Hybrid incompatibilities in the context of phylosymbiosis are resolved by hologenomic principles and exemplify this emerging postmodern synthesis.

The hologenome—the entire collection of nuclear, cytoplasmic, and microbial genes in animals and plants—brings forth several underappreciated modes of variation and evolution (*1*). In particular, genetic variation in the hologenome arises by changes in the nucleus, cytoplasm, and microbiome that can jointly encode phenotypes essential to health and fitness. Within the context of multilevel selection theory in evolution, the hologenome is a unit of selection backed by a vast body of empirical data (*2*). The recognition of the importance and universality of this multigenomic nature of animals is initiating a profound reassessment of our nuclear centric view of the life sciences (*3*), including the origin of species (*4*).

Hologenomic speciation, which we did not define in (*5*), occurs when both the host genome and microbiome cause reproductive isolation. We previously collated the evidence for this speciation process across plants and animals and found that the total evidence places symbiosis squarely into nearly all canonical speciation mechanisms (*6*). In this context, there are as many known microbes causing reproductive isolation as nuclear genes (*6*). Thus, hologenomic speciation is not a fringe example of hybrid incompatibility evolution, as described by Chandler and Turelli (*7*), but rather a sweeping framework for studying the basis of any reproductive isolation mechanism, including examples of animal sexual isolation and plant hybrid necrosis.

Adding to the synthesis of nuclear and microbial studies in speciation, we demonstrated that hybrid lethality, for the first time, is conditional on negative intergenomic epistasis between the nuclear genome and host-associated microbiome (*5*). Moreover, we introduced and defined the term "phylosymbiosis" to denote the pattern when host-associated microbial communities hierarchically cluster into dendrograms that recapitulate the phylogenetic relationships of host nuclear sequences, such as in wasps, hydra, ants, and hominids (*5*, *8*–*11*). This phylosymbiosis hypothesis differentiates random from deterministic assembly mechanisms of host-associated microbiotas and hence is a diversity-based metric to infer coadaptation.

We make two principal conclusions about hybrid lethality in *Nasonia*. The first is that "severe hybrid lethality in larvae can also be due to gene-microbe interactions." This discovery is not in dispute. Hybrid lethality is conditional on genome-by-microbiome interactions. Moreover, the gut bacterial community is demonstratively beneficial in animals and is vitally required for *Nasonia* metamorphosis and survival (*12*). The second conclusion is that "the phylosymbiotic microbiota can be understood as an addition to the coadapted genomes of a host organism rather than an arbitrary amalgam." The latter part of this conclusion is the focus of the commentary. Coadaptation is substantiated not only by phylosymbiosis but also by the evidence that the vital interdependencies of the genome and microbiome within *Nasonia* are in negative epistasis in hybrids—mirroring widespread descriptions of coadaptation between the nucleus and cytoplasmic organelles that break down in hybrids through intergenomic epistasis (*13*), including in *Nasonia* (*14*). This cytonuclear analogy is particularly informative to hologenomic speciation because it highlights the continuum of symbiotic interactions that occur from bacterial-derived organelles to host-associated bacteria.

Chandler and Turelli (*7*) advocate that the evidence for coadaptation is also compatible with hybrid breakdown to any bacteria, irrespective of the history of the host and microbe. Yet, with respect, we must point out that this opinion is based on claims that are factually inaccurate. We only inoculated bacteria that were isolated from or taxonomically found in *Nasonia* (*5*, *8*), including the *Escherichia coli* deemed as foreign. Resident microbes are important in interpreting the nature of hologenomic hybrid breakdown and no less relevant than understanding cytonuclear intergenomic incompatibilities (*13*) involving the resident mitochondria. Foreign microbes may cause hybrid lethality under hologenomic evolution (Fig. 1B, model vi), but so may foreign mitochondria or genes. Hence, the claim that any free-living and foreign bacteria to *Nasonia* can cause hybrid mortality is empirically unjustified and holds the ambiguous equivalency that any foreign mitochondria from different animals could cause mortality. The latter is plausible, but such experiments are dubious because they would not substantiate or refute coadaptation.

Nasonia evolved to handle their resident microbiota—namely, a finite subset of the most abundant and genetically diverse organisms on the planet, rather than all microbes. Hologenomic evolution is based on this intrinsic epistasis because the vital and synergistic interactions between host and microbiota are akin to those between any essential nuclear gene and another. Thus, although nuclear hybrid incompatibilities focus on intrinsic epistasis that breaks down in hybrids (Fig. 1A), intrinsic genome-by-microbiome interactions do the same and increase the number of potential hybrid incompatibilities (Fig. 1B) that can accelerate the evolution of hybrid breakdown (*6*). Our study is the first to show that hologenomic hybrid mortality is reversible under germ-free rearing (Fig. 1C). Finally, we certainly agree with the suggestion that a cross-species transformation experiment of the gut microbiota would be additional evidence for coadaptation; we proposed it to Chandler and Turelli in a previous dialogue. However, the weakness in overstating this experiment is that any speciation gene in animals would have the same experimental flaw. Such studies are typically not done in practice. In regard to coadaptation, there is no evidence or concept that supports the authors' opinion in the Technical Comment.

As previously described (*6*), phylosymbiosis does not presume that microbial communities are vertically transmitted or cospeciate with their hosts; rather, host species acquire microbiota that are more similar within host species than between related host species in each generation. Use of 16*S* sequencing, quantitative real-time fluorescence polymerase chain reaction, and florescent in situ hybridization confirm that *Nasonia* microbiota structures are conserved within individuals and thus are repeatable across replicates within a generation (*8*). We present phylosymbiosis in this context as a deterministic pattern of microbiota assembly that is unlikely to arise by neutral processes of dispersal limitation and

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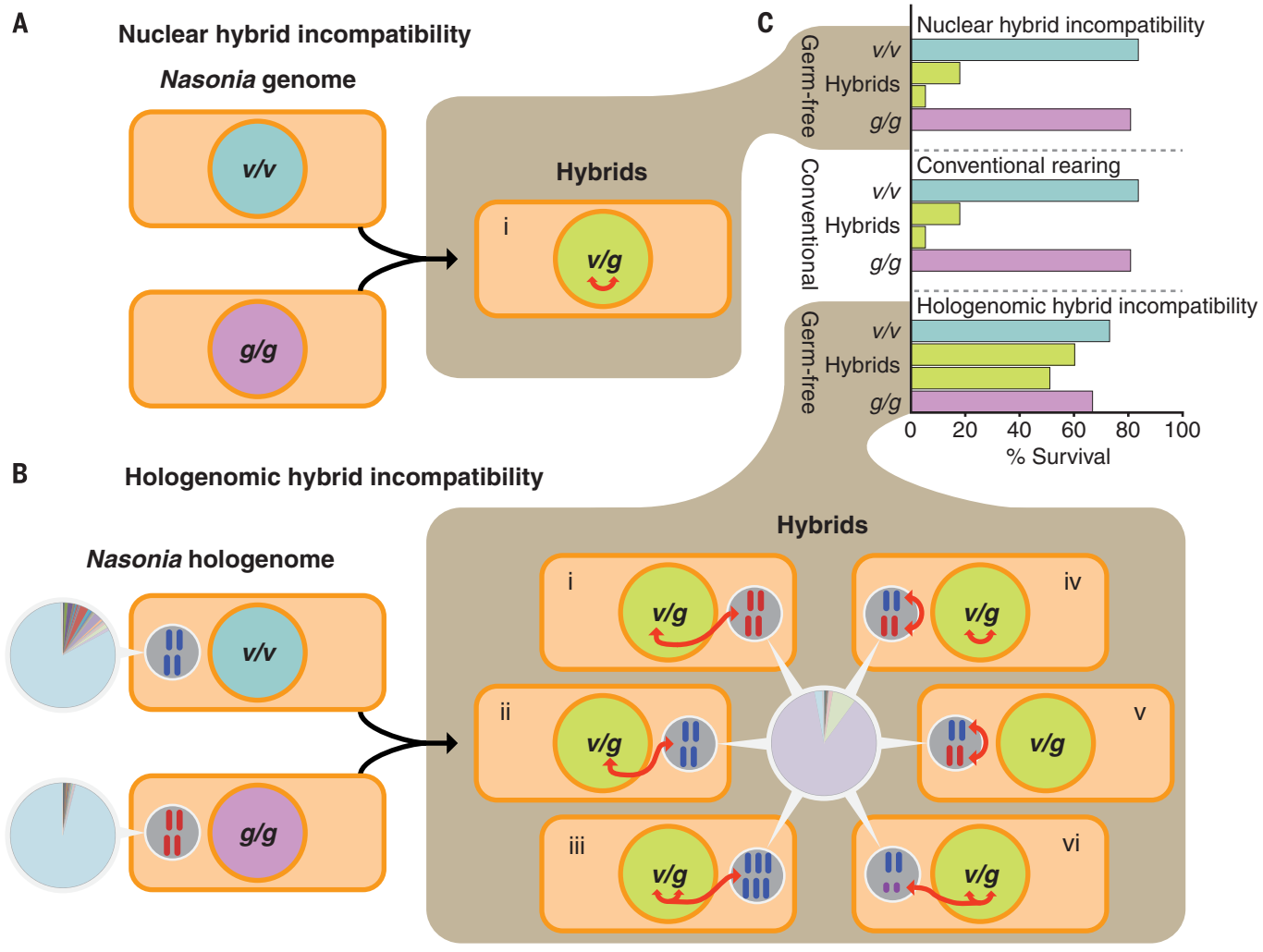

Fig. 1. Evolutionary genomics of hybrid incompatibilities. (**A**) A nuclear-centric model of hybrid incompatibilities in which red arrows indicate potential negative epistasis between *N. vitripennis* (*v*/*v*) and *N. girault*i (*g*/*g*) genes. (**B**) Six hologenomic models of hybrid incompatibility evolution in which pie charts reflect microbiota data published in (*5*). The *Providenica* taxa in parental *v* and *g* encompass several different 16*S* oligotypes, and *Proteus*, the dominant microbe in hybrids, is present at low abundance in parental *v* and *g*. (i) The *v* genome negatively interacts with the *g* microbiome. (ii) The *g* genome negatively interacts with the *v* microbiome. (iii) The *v*, *g*, and overgrown resident microbiomes interact together. (iv) The *v* and *g* microbiomes and derived genomes negatively interact with each other, respectively. (v) A microbiome-microbiome interaction detrimental to the host because the two bacterial genotypes are incompatible with each other (i.e., cytoplasmic incompatibility). (vi) The *v* and *g* genomes negatively interact with a new bacterial species (purple); alternatively, the two genomic interactions can abnormally suppress beneficial bacteria and lead to hybrid problems due to the lack of beneficial bacteria, such as an autoimmune response. (**C**) The predicted rescue of nuclear-centric and hologenomic hybrid incompatibilities in germ-free versus conventional rearing conditions. Portions of this figure are discussed in previous modeling of Bateson-Dobzhansky-Muller models of hybrid incompatibility with and without microbes (*6*).

disturbance (*15*). Thus, phylosymbiosis is a diversity-based metric to infer coadaptation. The issue was of such central focus for Chandler and Turelli that they reanalyzed the data with curious methods that restrain high bootstrap support [figure 1 in (*7*)] or yield an alternative dendrogram [figure 2 in (*7*)] for *Nasonia* phylosymbiosis. We discuss these misapplications below.

First and most important, they use a UniFrac bootstrapping scheme that reduces their analysis to such low sampling depth (rarefied to 206 reads per sample) that only poor values are obtained (Fig. 2A). The low sampling depth originates from the inclusion of *Sarcophaga bullata*, pupal fly hosts of *Nasonia*, as the outgroup in the dendrograms. The low sequencing depth of *S. bullata* is biological because the concentrations of insect DNA for each sample were normalized before the 16*S* amplification. A separate concern is that *S. bullata* last shared a common ancestor with *Nasonia* ~*355* million years ago, and microbiota variation turns over too quickly to obtain a phylosymbiotic signal between distantly related animals (*6*, *16*). We included this taxa in our original study not to examine phylosymbiosis but to represent the host environment from which *Nasonia* larvae acquire their microbiota. Finally, the inclusion of *S. bullata* in their dendrogram analysis forces an anomalous comparison between the microbiota of the pupae of this fly species and the larvae of *Nasonia*, despite knowledge of microbial successions over *Nasonia* development (*8*). It is important to note that despite these defects, Chandler and Turelli still replicate a supported phylosymbiotic dendrogram of the *Nasonia* microbiota in their figure 1. In addition, by implementing a bootstrap analysis without *S. bullata*, we show robust bootstrap support for *Nasonia* phylosymbiosis (Fig. 2). Thus, their claim for weak support of phylosymbiosis is misguided.

Second, Chandler and Turelli performed a microbiota analysis with bacterial operational taxonomic units (OTUs) clustered at a 95% similarity cutoff and taxonomy assigned at 97% pairwise identity compared with our published 97% OTU default clustering and 95% pairwise identity taxonomy assignment. This error deflates both the number of potential OTUs for each sample and

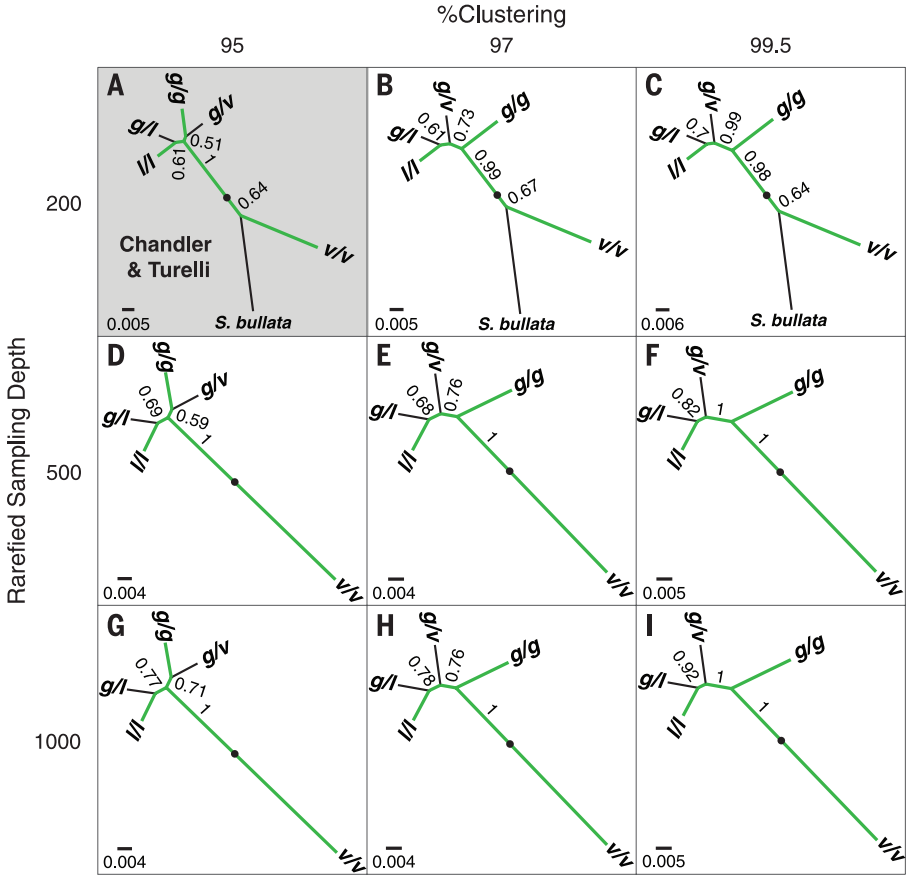

Fig. 2. Separating the effects of sampling depth and OTU clustering on phylosymbiotic accuracy. Weighted UniFrac, unweighted pair group method with arithmetic mean (UPGMA) cluster analyses at 95, 97, and 99.5 percent OTU clusterings, and rarefied sampling depths of 200, 500, and 1000 sequences (75% of smallest sample size, rounded). (**A**) Weighted UniFrac UPGMA generated using the parameters provided by Chandler and Turelli. (**B**) Weighted UniFrac UPGMA generated using the parameters specified in (*5*). (**C**) Weighted UniFrac UPGMA generated using the parameters specified in (*11*). (**D** to **I**) Weighted UniFrac UPGMA generated with aforementioned parameters under different, rarefied sampling depth (500 sequences ~50% of smallest library and 1000 sequences ~75%) that eliminates the *S. bullata* host as an inappropriate outgroup. All jackknife support values are calculated in UniFrac based on a midpoint root of the longest branch (black circle). We preferentially draw the dendrograms as unrooted because there is no basis for rooting community cluster dendrograms of microbiomes. Green branches emphasize phylosymbiotic and phylogenetic relationships of *Nasonia* in which *g/g* (*N. girault*i) and *l/l* (*N. longicornis*) are more closely related to each other than to *v/v* (*N. vitripennis*).

the bootstrap support of the dendrogram, as exemplified when we serially increase the cluster cutoff (Fig. 2, A to C).

Third, they curiously dismiss the UniFrac cluster analysis, a widespread tool designed for comparing microbial community relationships. The authors conduct an alternative cluster analysis that does not take into account the phylogenetic relationships of the bacterial taxa [figure 2 in (*7*)]. The net effect is to reduce the taxonomic characters for undefined reasons. Moreover, the key paper that Chandler and Turelli cite as confident support for phylosymbiosis (*11*) exhibits the same kind of "weak support" asserted for our original analysis—namely, lack of bootstrap values and method sensitivity to producing phylosymbiotic trees. Serious consideration of why reconstruction methods differentially recover phylosymbiosis demonstrates that there is biological meaning, rather than subjective weakness, in the variation (*10*).

The issues at hand are not just technical issues but matters critical to the nature of biology itself. There is an intellectual gulf between the position that the nuclear genome is a singular unit of selection and the holistic view that an organism's summed genetics determines its phenotype and is thus an underappreciated target of selection. In sum, biology has entered a new era (*2*–*4*) with the capacity to understand that an organism's genetics and fitness are inclusive of its microbiome; we invite the community of biologists to join the debate as the horizon unfolds.

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