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A conceptual framework for host-associated microbiomes of hybrid organisms

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Abstract

1. Hybridization between organisms from evolutionarily distinct lineages can have profound consequences on organismal ecology, with cascading effects on fitness and evolution. Most studies of hybrid organisms have focused on organismal traits, for example, various aspects of morphology and physiology. However, with the recent emergence of holobiont theory, there has been growing interest in understanding how hybridization impacts and is impacted by host-associated microbiomes. Better understanding of the interplay between host hybridization and host-associated microbiomes has the potential to provide insight into both the roles of host-associated microbiomes as dictators of host performance as well as the fundamental rules governing host-associated microbiome assembly. Unfortunately, there is a current lack of frameworks for understanding the structure of host-associated microbiomes of hybrid organisms.
2. In this paper, we develop four conceptual models describing possible relationships between the host-associated microbiomes of hybrids and their progenitor or 'parent' taxa. We then integrate these models into a quantitative '4H index' and present a new R package for calculation, visualization and analysis of this index.
3. We demonstrate how the 4H index can be used to compare hybrid microbiomes across disparate plant and animal systems. Our analyses of these data sets show variation in the 4H index across systems based on host taxonomy, host site and microbial taxonomic group.
4. Our four conceptual models, paired with our 4H index and associated visualization tools, facilitate comparison across hybrid systems. This, in turn, allows for systematic exploration of how different aspects of host hybridization impact the host-associated microbiomes of hybrid organisms.

KEYWORDS
Aitchison simplex, holobiont, host-associated microbiome, hybrid, R package

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1 | INTRODUCTION

Hybridization is increasingly recognized as an important component of ecological and evolutionary processes. Consequences of hybridization span the fitness spectrum ranging from infertility and death (Brucker & Bordenstein, 2013; Zhang et al., 2014) to innovation and adaptation (Abbott et al., 2013; Dowling & Secor, 1997; Patton et al., 2020; Seehausen, 2004). Ultimately, these fitness consequences dictate the role that hybridization plays in the success or failure of different genetic lineages (Seehausen, 2004; Pala & Coelho, 2005; Larouche et al., 2020; Todesco et al., 2016). If, for example, hybridization produces sterile offspring, then it can drive the emergence of genetic sinks and evolutionary dead ends (Tripp & Manos, 2008) and thus serve as a 'brake' for evolution. Alternatively, if hybridization facilitates ecological release and/or sexual isolation (either directly through mating barriers or indirectly through altered temporal or spatial proximity), then it can promote lineage diversification and thus serve as a 'motor' for evolution (Heard & Hauser, 1995).

Most early research on hybrid organisms focused on understanding how hybridization impacts host fitness through effects on host traits, for example, fecundity (Campbell et al., 2006; Dobzhansky, 1934; Forejt, 1996; Hovick & Whitney, 2014; Reed & Sites Jr, 1995), physiology (Brown & Bouton, 1993; Cooper & Shaffer, 2021; Lafarga-De la Cruz et al., 2013; Martins et al., 2019; Pereira et al., 2014), morphology (Capblancq et al., 2020; Carreira et al., 2008; Jackson, 1973; Mérot et al., 2020) and behaviour (Robbins et al., 2010, 2014). Recently, however, there has been growing recognition that macroorganisms are not autonomous units. Rather, they are collectives or 'holobionts' compromised of both a host and all of its host-associated (HA) microbes (Baedke et al., 2020; Bordenstein & Theis, 2015; Bosch & Miller, 2016; Margulis & Fester, 1991). Thus, just as it is important to understand how hybridization impacts the traits of the host, it is equally important to understand how hybridization impacts the traits of the holobiont, including characteristics of the HA microbiome (Miller et al., 2021). Indeed, the eco-evolutionary basis for holobionts has led to entirely new branches of research in areas as diverse as human health (Postler & Ghosh, 2017; Walter et al., 2013), conservation (Bahrndorff et al., 2016; Banerjee et al., 2020; Carthey et al., 2020; Jiménez & Sommer, 2017; Jin Song et al., 2019; Maebe et al., 2021; Redford et al., 2012; Trevelline et al., 2019; West et al., 2019; Zhu et al., 2021) and biotechnology (Bredon et al., 2020; Ren et al., 2022), and it is currently poised to do so within the field of hybridization research as well.

The importance of the holobiont concept stems from the many host traits and processes that are either partially or fully dependent on host-associated microbes (Fontaine & Kohl, 2020; Friesen et al., 2011; Nobs et al., 2019; Walters et al., 2020). As an example, gut microbiomes are strong regulators of host metabolic phenotype (Claus et al., 2008; Li et al., 2008; Mayneris-Perxachs et al., 2016). This, in turn, impacts host energy balance (Corbin et al., 2020; Nieuwdorp et al., 2014; Xifra et al., 2019), including both energy intake as well as use and expenditure. Gut microbiomes can also be important determinants of dietary niche (Blyton et al., 2019; Greene et al., 2020; Heys et al., 2021; Kohl et al., 2014; Moeller & Sanders, 2020), either by provisioning hosts with key nutrients (Hu et al., 2018; Jing et al., 2020; Ju et al., 2020) or by detoxifying defensive compounds found in host food sources (Zheng et al., 2016). Beyond diet and metabolism, HA microbiomes influence a range of other host traits as well (Archie & Theis, 2011; Bravo et al., 2011; Davidson et al., 2018; Ezenwa et al., 2012; Gaona et al., 2016; Grinberg et al., 2022; Jia et al., 2021; Kirchoff et al., 2019; Neufeld et al., 2011; Sampson & Mazmanian, 2015; Sharon et al., 2010). Healthy gut (Chen et al., 2018; Kamada et al., 2013), skin (Chen et al., 2018; Harris et al., 2006; Kueneman et al., 2014) and vaginal microbiomes (Brotman et al., 2010), for example, provide pathogen resistance across a broad spectrum of animal species (Buffie & Pamer, 2013; Ubeda et al., 2017; Woodhams et al., 2016). Indeed, amphibian skin microbiomes have been extensively studied as a means of defending hosts from devastating fungal pathogen (*Batrachochytrium dendrobatidis* and *B. salamandrivorans*) epidemics (Bates et al., 2018, 2022; Rebollar et al., 2016, 2020). In humans, disruptions to healthy HA microbiomes also underly a range of non-infectious diseases (Ahn et al., 2013; Zackular et al., 2013) such as rheumatoid arthritis (Bergot et al., 2019; Scher & Abramson, 2011) and irritable bowel syndrome (Chong et al., 2019; Pimentel & Lembo, 2020). Ultimately, the cascading effects of HA microbiomes on host traits and processes—ranging from host energy balance and dietary niche through disease risk and immune dysfunction—have strong consequences on host ecological success (Abbott et al., 2021) and, by extension, host evolution (Kolodny et al., 2020; Opstal, & Bordenstein, 2015; Zilber-Rosenberg & Rosenberg, 2008).

Although there has been substantial literature documenting both coevolutionary (Ehrlich & Raven, 1964; Janz, 2011; Janzen, 1980; Thompson, 1994, 2005) processes and codiversification patterns (Janz, 2011; Nishida & Ochman, 2021; Suzuki et al., 2022; Thompson, 1989) between hosts and their HA microbiomes (Apprill et al., 2020; Chiarello et al., 2018; Ley et al., 2008; Meadows, 2022; Moran & Sloan, 2015; Ochman et al., 2010; Phillips et al., 2012; Sanders et al., 2014; Scheelings et al., 2020; Walker et al., 2019), the study of how HA microbiomes respond when divergent host lineages reunite, or admix, through hybridization is relatively new (Malukiewicz et al., 2019). One of the earliest investigations into hybrid microbiomes was in *Nasonia* wasps (Brucker & Bordenstein, 2013). In this system, up to 90% lethality is observed in F_2 males of *N. vitripennis*/*N. giraulti* crosses. However, rearing wasps under germ-free conditions results in near complete rescue of the same F_2 males. This suggests a microbial basis to hybrid lethality. Interestingly, the 10% of hybrid *N. vitripennis*/*N. giraulti* males that survive under natural conditions exhibit highly transgressive microbial phenotypes. This includes both the appearance of novel microbial taxa in hybrid microbiomes as well as shifts in the abundances of microbial taxa that are shared among parents and hybrids.

More recent studies on hybrid vertebrates paint a similar picture. For example, hybrid house mice (*Mus musculus musculus* and *Mus m. domesticus*) in central Europe (Wang et al., 2015) exhibit widespread

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transgressive microbiomes. Furthermore, like the *Nasonia* wasp system, there is evidence that the altered microbial phenotypes of hybrid individuals at least partially explain their poor fitness outcomes (Baird et al., 2012; Britton-Davidian et al., 2005; Forejt & Iványi, 1974; Good et al., 2008; Sage et al., 1986; Turner et al., 2012). In particular, there is an interaction between inflammation, immune gene expression and the gut microbiome that appears to cause hybrid mice to exhibit defects in immunoregulation. This may be one reason why hybrid individuals are restricted to a narrow tension zone where the two parent subspecies co-occur (Balard & Heitlinger, 2022; Barton & Hewitt, 1985). A range of additional studies, including hybridization of sika deer (*Cervus nippon*) and elk (*Cervus elaphus*) (Li et al., 2016), lake whitefish lineages (*Coregonus clupeaformis*) (Sevellec et al., 2019), blunt snout bream (*Megalobrama amblycephala*) and topmouth culter (*Culter alburnus*) (Li et al., 2018) and desert (*Neotoma lepida*) and Bryant's (*Neotoma bryanti*) woodrats (Nielsen et al., 2023) have reiterated the finding that hybrid animals often exhibit altered microbiomes relative to their progenitors (i.e. 'parent' lineages or 'parent' taxa). Indeed, even beyond the animal kingdom, hybrid macroorganisms are commonly associated with perturbations to the HA microbiome (Cregger et al., 2018; O'Brien et al., 2019; Wagner et al., 2020).

As suggested above, the study of hybridization and its impact on HA microbiomes is important for understanding host fitness and evolution (Baeckens, 2019; Muñoz & Bodensteiner, 2019). However, even beyond host success, hybrid systems are of interest because they facilitate an understanding of genotype–phenotype interactions (Kearney, 2005; Kratochwil & Meyer, 2015). Many hybrid zones (Cooper & Shaffer, 2021; Robbins et al., 2010; Walls, 2009), especially systems where F_2 individuals readily admix with their progenitors, provide variable genetic combinations (Lee et al., 2017; Pfennig, 2021) and degrees of heterozygosity across hybrid individuals. Consequently, these systems serve as natural laboratories for understanding how host genetics and environmental characteristics influence host traits. For example, in a study investigating how host genetics and the environment impact HA microbiomes across a *Neotoma* woodrat hybrid zone, Nielsen et al. (2023) demonstrated that HA microbial composition was predominately driven by host genetics (genotypic classes), while HA microbial richness was predominately driven by the environment (core diet + vegetation communities). Applying similar approaches to other hybrid systems may be a fruitful avenue for disentangling the long-standing nature versus nurture paradigm as it applies to HA microbiomes and HA microbiome assembly.

Despite the increasing recognition that HA microbiomes are an important facet of hybridization and that hybrid organisms are valuable systems for understanding HA microbiome structure and function, there is a lack of frameworks for describing and comparing hybrid HA microbiomes across the tree of life. In this paper, we develop four conceptual models delineating potential relationships between hybrid microbiomes and the microbiomes of their progenitors. We discuss the underlying implications of each model, how each model might arise based on fundamental host mechanisms and how each model could impact host fitness. We then integrate these four models into a quantitative '4H index' that can be used to assess the relative importance of each model across widely disparate hybrid systems. Finally, we introduce an R package, HybridMicrobiomes (https://cran.r-project.org/web/packages/HybridMicrobiomes/index.html), containing a series of functions that allow researchers to apply the 4H index to their own hybrid microbiome data sets.

2 | MATERIALS AND METHODS

2.1 | Conceptual models

We propose four conceptual models—the Union Model, the Intersection Model, the Gain Model and the Loss Model—to describe the potential relationships between the HA microbiomes of hybrid individuals and those of their progenitors (see Figure 1). These four

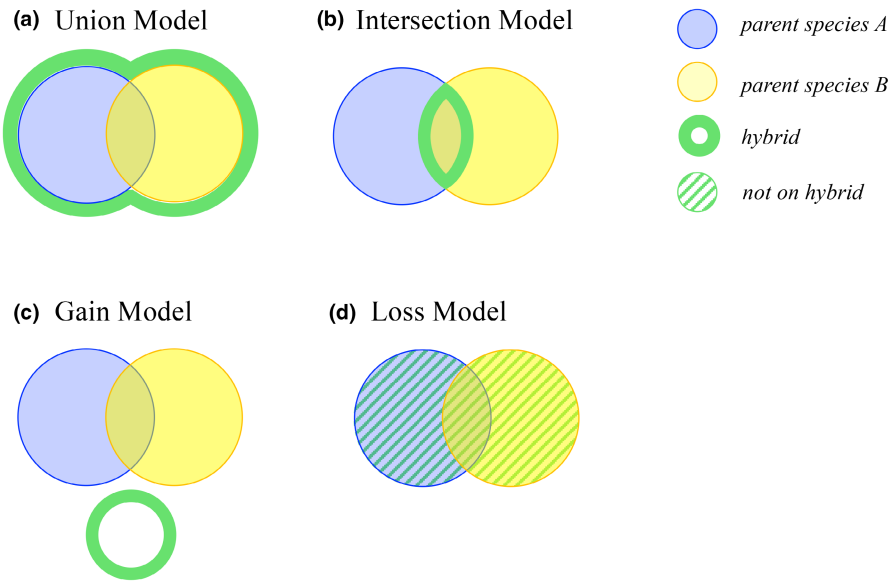

FIGURE 1 The limiting scenarios for each of our four conceptual models describing the host-associated microbiomes of hybrid organisms. Hybrid organisms can (a) host all of the microbial taxa found on *either* progenitor (Union), (b) host only those microbial taxa found on *both* progenitors (Intersection), (c) host only novel microbial taxa found on *neither* progenitor (Gain) or (d) be missing all microbial taxa found on one or both progenitors (Loss). Note that, in the final scenario, assuming that there is no gain of microbial taxa, the hybrid has no microbiome at all.

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models represent extreme or limiting scenarios, each portraying an idealized relationship between hybrid and progenitor HA microbiomes. Realistic hybrid microbiomes can then be described as differing combinations of these four idealized models. In what follows, we delineate the four models, discuss possible underlying mechanisms and outline their potential for impacting hybridization outcomes. For the sake of simplicity, we introduce each of the models within the framework of microbiome membership (taxon incidence) rather than composition (taxon abundance). However, comparable arguments can be made for abundance relationships between progenitor and hybrid microbiomes as well.

2.1.1 | The Union Model

In its most extreme form, this model implies that hybrid microbiomes are comprised of all microbial taxa present on at least one progenitor and nothing else (see Figure 1a). This could occur if carrying a particular host genome fosters colonization by associated microbial taxa. Notably, such fostering could emerge either directly through host interactions with the microbe (e.g. if specific hybrid and/or progenitor morphologies provide housing for symbiotic microbes (Belcaid et al., 2019; Delaux & Schornack, 2021; Fronk & Sachs, 2022)) or indirectly through effects on host behaviour or ecology (e.g. if hybrids colonize a progenitor's environment and subsequently acquire environmental microbes). To the extent that hybrid individuals share genetic material from both progenitors (note that this may vary depending on the extent of back-crossing), hybrids should support all microbes present on either progenitor. Said differently, in the Union Model, the host genome acts as a 'ticket' for acquiring a particular microbiome. Having two tickets (i.e. each representing a unique genomic component) results in the acquisition of two microbiomes, one from each progenitor.

Hybrids characterized exclusively by the Union Model (see Figure 1a) should have more taxonomically diverse microbiomes than either progenitor. Importantly, greater taxonomic diversity could result in greater functional diversity as well (Petchey & Gaston, 2002), with important consequences for host health and ecological performance. Consider a thought experiment wherein two different insect species are each limited to a distinct set of host plants based on the need for gut microbial detoxification of plant defensive compounds. If the hybrid offspring of these two insect species harbour the gut microbiomes of both progenitors, then hybrid microbiomes should be able to detoxify both sets of host plants, allowing hybrids to utilize all resources open to either progenitor. More broadly, greater functional capacity of hybrid microbiomes could enable hybrids to persist in habitats that are intermediate to their progenitors or across all habitats colonized by either progenitor. Beyond expanded function, a more diverse hybrid microbiome may have other benefits as well. Although contentious, both in microbiome (Deng, 2012; He et al., 2013; Wagg et al., 2018) and general ecology literature, diversity (Ives & Carpenter, 2007; McCann, 2000) has long been associated with lower temporal variability and increased resistance to invasion (McCann, 2000). If this is true for HA microbial communities, then hybrids following the Union Model may gain the advantage of having a more resilient microbiome that is more resistant to colonization by pathogens (Harrison et al., 2019).

However, there are likely costs to the Union Model as well. Most obvious are the challenges of bringing together large numbers of distinct microbial taxa from different progenitors. Consider Bateson–Dobzhansky–Muller (BDM) incompatibilities (Muller, 1942; Orr, 1995; Orr & Turelli, 2001), which emerge in hybrid organisms due to mismatches between the genes from their two progenitors. If BDM incompatibilities are a common outcome of combining different progenitor genomes, then analogous mismatches that result from combining different microbial metagenomes should also be possible. Furthermore, there could be mismatches between the microbial metagenome from one progenitor and the host genome from the other, as outlined in the 'microbial-assisted BDM' model by Brucker and Bordenstein (2012). Indeed, if BDM incompatibilities scale with genome size (a tenuous assumption), host–microbe and microbe–microbe incompatibilities may be more likely than traditional BDM incompatibilities simply because the microbial metagenome is typically much larger than the genome of the host itself. Whether or not this is the case, the *Mus musculus* and *Nasonia* systems suggest that microbial-assisted BDMs are certainly a possibility among hybrid HA microbiome systems.

2.1.2 | The Intersection Model

In its most extreme form, this model suggests that hybrid microbiomes are comprised of all microbial taxa simultaneously present on both progenitors and nothing else (see Figure 1b). Note that, within the framework of our conceptual models, the Intersection Model is a subset of the Union Model (i.e. the Union Model comprises all microbial taxa found on one or both progenitors, while the Intersection Model comprises only those microbial taxa found on both progenitors). We describe our conceptual models in this way because it best reflects potential mechanisms by which Union and Intersection of HA microbiomes may emerge. However, a slightly different definition of the Union model (not including microbial taxa found on both progenitors) is used for the 4H index. This is done to avoid double counting components of microbial diversity (see Section 2.2). Further note that, unlike the Union Model, a hybrid cannot be exclusively characterized by the Intersection Model unless there are no microbial taxa unique to one of the two progenitors. This is because a hybrid that only harbours microbial taxa found on both progenitors must, in addition, have lost all microbial taxa found on only one of the two progenitors. The inter-relatedness of the Intersection and Loss models is discussed more below.

The Intersection Model could occur if a particular host genome hinders or prevents colonization by unassociated microbial taxa. Again, the underlying mechanism could be direct (e.g. changes in the host immune system) or indirect (e.g. changes in host behaviour that alter exposure to environmental microbes). In either case, hybrids

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that carry genetic material from both progenitors will be more refractory to, or isolated from, a wider range of microbial taxa. Said differently, in the Intersection Model, each host genome acts as a 'gate'. Having two gates blocks a wider range of microbes, leaving only those taxa that are permitted access by both progenitors. Thus, hybrids characterized by the most extreme form of the Intersection Model (see Figure 1b) should have less taxonomically diverse microbiomes which could have consequences for functional diversity as well. For instance, in our previous insect example (see 'The Union Model'), the Intersection Model could leave hybrids without the ability to detoxify either set of progenitor host plants, placing a substantial limitation on feeding opportunities. This, in turn, could have impacts on fitness, leading to higher rates of starvation, underperformance due to toxin build-up or even poisoning directly. Similar negative effects on survival could be possible due to more general mechanisms associated with microbial diversity as well, for example, the loss of microbiome stability and pathogen resistance. The benefit of the Intersection Model, of course, is that it virtually eliminates opportunities for microbe–host or microbe–microbe incompatibilities. This is because, in the Intersection Model, all microbe–host and microbe–microbe interactions that occur on the hybrid are already present on both progenitors.

2.1.3 | The Gain Model

In its most extreme form, this model suggests that hybrid microbiomes only include microbial taxa not present on either progenitor (see Figure 1c). Like the Intersection Model, a hybrid cannot be exclusively characterized by the Gain Model unless the progenitor microbiomes are fully devoid of microbial taxa. Again, this is because a hybrid that only harbours novel microbial taxa must, in addition, have lost all microbial taxa found on the two progenitors. The Gain Model is possible if HA microbiomes are idiosyncratically sensitive to specific gene combinations that arise from merging progenitor genomes. Broadly speaking, the Gain Model is the microbial equivalent of Bateson's 'saltational evolution' (Bateson, 1984, 2002) or Goldschmidt's 'hopeful monsters' (Goldschmidt, 1933, 1940). Like Bateson's and Goldshmidt's models, the Gain Model posits that hybridization can yield profound (saltational) changes in phenotype (Theißen, 2006, 2009), and that these phenotypic changes may enable hybrids to establish an entirely novel ecological niche relative to their progenitors (Dittrich-Reed & Fitzpatrick, 2013; Goldschmidt, 1933; Mallet, 2007). However, unlike Bateson and Goldschmidt, who focused on host genes, the Gain Model assumes that there are underlying microbial dimensions to the saltational change. Arguably, adding microbial dimensions provides even more opportunity for saltational change, again because of the vast size and diversity of functions encompassed by the microbial metagenome relative to the host genome itself. Once more, consider our hypothetical insect example (see 'The Union Model'). Hybrid insects characterized by the most extreme form of the Gain Model should harbour an entirely new set of gut bacteria with novel taxa and potentially different detoxification properties as compared to their progenitors. Thus, rather than being able to use both of their progenitor's host plants (Union Model) or neither of their progenitor's host plants (Intersection Model), these 'saltational' hybrids could potentially colonize an entirely novel set of host plants not used by either progenitor.

More so than the Union, Intersection or Loss models, the Gain Model is responsible for phenotypic novelty and thus, provides the building blocks for evolutionary innovation. This could result in rapid adaptation, escape from competition with their progenitors or even reproductive isolation. Indeed, in some cases, the Gain Model may actually accelerate speciation (Mallet, 2007). However, the Gain Model may have non- or maladaptive consequences as well. Notably, there is no a priori reason to believe that the acquisition of large numbers of novel microbial taxa will be generally beneficial to a host. In fact, there are many reasons to believe the opposite. In particular, the Gain Model describes a scenario of rapid evolutionary change (i.e. the introduction of novel microbial metagenomic content to the hybrid) that occurs far outside the confines of more typical host–microbe coevolutionary relationships forged over generations of symbiosis. As a result, the Gain Model exemplifies a 'high risk, high reward' scenario, and novel microbes acquired by the hybrid could just as easily enhance or reduce host fitness. Thus, like Goldschmidt's hopeful monsters, the Gain Model relies on 'happy accidents' (Ross, 1983-1994) meaning that many hybrid individuals are likely to fail for each ecological success.

2.1.4 | The Loss Model

In its most extreme form, this model suggests that hybrid microbiomes are missing all microbial taxa that are present on one or both progenitors and have gained no new microbial taxa (see Figure 1d). In other words, the most extreme form of the Loss Model implies that hybrids have no microbiome at all. Again, this is unrealistic. Thus, just as the Intersection and the Gain models cannot occur realistically independent of the Loss Model, nor can the Loss Model occur realistically independent of at least one or more of the other models. The non-independence of the various models reflects the fact that, except in very special and typically non-realistic scenarios (e.g. when the progenitors or hybrids have no HA microbes), realized systems will always be combinations of the idealized models. The idealized models, however, serve as limits that emerge out of various scenarios by which host genomes, and in particular hybrid host genomes, could feasibly impact HA microbiome assembly. As in the Gain Model, the loss of microbes present on both progenitors describes a 'saltational' scenario that is possible if HA microbiomes are idiosyncratically sensitive to gene combinations of the progenitors. In contrast to the Gain Model, however, the saltational change invoked by the Loss Model is the deletion, rather than the addition, of microbial taxa.

The Loss Model gives rise to hybrid microbiomes with lower overall diversity and potentially lower functional capacity as well.

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Indeed, as suggested above, in the limit of a hybrid organism exclusively characterized by the Loss Model, there would be no host-associated microbiome at all. Returning, for the last time, to our hypothetical insect complex (see 'The Union Model'), the Loss Model predicts that hybrid insects should lack microbes present on one or both progenitors. In the case of the latter, hybrids would lose the ability to detoxify host plants that are usable by both of their progenitors. Like the Intersection Model, this could limit opportunities for feeding, cause toxin build-up or result in poisoning of hybrid insects. Lower microbiome diversity could also lead to a suite of additional challenges like greater microbiome instability and lower pathogen resistance. Again, however, the costs of low diversity microbiomes may be balanced out by the benefits of reduced opportunities for host–microbe or microbe–microbe incompatibilities.

2.2 | The 4H index and quaternary plots

While the four conceptual models in Section 2.1 present limiting, extreme or idealized scenarios, any realistic hybrid system will almost certainly exhibit mixed support across two or more conceptual models. To examine the importance of each of the four conceptual models to any given hybrid system, we introduce the 4H index, along with R package HybridMicrobiomes (https://cran.r-project.org/web/packages/HybridMicrobiomes/index.html), which can be used to calculate and graph the 4H index for any hybrid system. The 4H index uses the 'core microbiomes' of each host class (where we use 'host class' to refer to any one of the three types of hosts—the first progenitor, the second progenitor or the hybrid—in a hybrid complex) to determine which microbial taxa are lost and gained on hybrid organisms relative to their progenitors.

To define the core microbiome, we use a tunable parameter, ρ, which can range from $\rho = 1$ (i.e. microbial taxa are only considered if they are present on every host of a particular host class) to $\rho = 0$ (i.e. the full microbiome; all microbial taxa are considered regardless of the number of hosts they are found on). Consistent with the common definition of a core microbiome, we typically select higher values of ρ. This is based on the assumption that microbial taxa with strong consequences for host ecology and/or evolution should be detectable on the majority of hosts within a population. However, researchers who have reason to suspect otherwise can use a lower value of ρ or can compare the 4H index across a range of ρ values (see Figures S2.1–S2.4; Tables S2.1 and S2.2).

While core microbial taxa are usually defined as those present on a threshold number of hosts, alternate definitions exist that incorporate microbial abundances as well (Shade & Stopnisek, 2019). To allow for this, we include a second threshold, ϑ, based on the average relative abundance (across all hosts within a class) that a microbial taxon must reach to be considered part of the core. In addition, we include a third threshold, ε, based on the minimum relative abundance that a microbial taxon must reach on at least one host to be considered part of the core. By default, the HybridMicrobiomes R package sets both $\vartheta = 0$ and $\varepsilon = 0$. However, researchers who would like to consider abundance thresholds have the option to do so. Thus, both the 4H index and the HybridMicrobiomes R package provide flexibility that can be decided within the context of a particular hybrid system (though a common set of parameters should be used for any comparison *between* hybrid systems).

For the 4H index, ρ, ϑ and ε are the same across all host classes. However, each host class (i.e. each progenitor and the hybrid) is separately assigned its own core microbiome. Thus, a microbial taxon is part of a host's core microbiome provided it is found on at least ρN hosts of that host class at a minimum average abundance of ϑ and a minimum abundance on at least one host of ε, where N is the number of hosts of each class and should be the same across all host classes (i.e. a balanced design with equal numbers of each progenitor and the hybrid; note that the HybridMicrobiomes package includes bootstrapping steps that will downsample data sets such that a balanced design is achieved). In what follows, we describe four versions of the 4H index, two based on incidence of microbial taxa and two based on abundance of microbial taxa.

2.2.1 | Incidence-based analyses

Our two incidence-based methods are inspired by the Jaccard index (Jaccard, 1908) and the Sorensen index, respectively (Dice, 1945; Sorensen, 1948). For any given ρ, ϑ and ε, we define P_1, P_2 and H as the set of core microbial taxa present on the first progenitor, the second progenitor and hybrids. We then determine the number of microbial taxa shared by different combinations of hybrid and progenitor classes. Specifically, we define:

$$a = \left|(P_1 \cap P_2) \cap H\right|, \tag{1a}$$

$$b = \left|(P_1 \cup P_2) \cap H\right| - a, \tag{1b}$$

$$b_1 = \left|P_1 \cap H\right| - a,$$

$$b_2 = \left|P_2 \cap H\right| - a,$$

$$c = |H| - a - b, \tag{1c}$$

$$d = \left|P_1 \cup P_2 \cup H\right| - a - b - c, \tag{1d}$$

$$d_1 = \left|P_1\right| - \left|P_1 \cap P_2\right| - \left|P_1 \cap H\right| + a,$$

$$d_2 = \left|P_2\right| - \left|P_1 \cap P_2\right| - \left|P_2 \cap H\right| + a,$$

$$d_{12} = d - d_1 - d_2.$$

In Equation (1), $|S|$ denotes the cardinality of set S, where S is any set. Accordingly, a is the number of microbial taxa shared by both progenitors and the hybrid, b is the number of microbial taxa shared by one progenitor (but not both) and the hybrid, b_1 is the number of microbial

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taxa shared by the first progenitor and the hybrid, b_2 is the number of microbial taxa shared by the second progenitor and the hybrid, c is the number of microbial taxa found only on the hybrid, d is the number of microbial taxa found only on one or both progenitors, d_1 is the number of microbial taxa found only on the first progenitor, d_2 is the number of microbial taxa found only on the second progenitor and d_{12} is the number of microbial taxa found only on both progenitors. For the Jaccard-inspired method, we define the four dimensions of the 4H index (three independent dimensions) as:

$$\mathcal{U} = \frac{b}{a+b+c+d}, \tag{2a}$$

$$\mathcal{U}_1 = \frac{b_1}{a+b+c+d},$$

$$\mathcal{U}_2 = \frac{b_2}{a+b+c+d},$$

$$\mathcal{I} = \frac{a}{a+b+c+d}, \tag{2b}$$

$$\mathcal{G} = \frac{c}{a+b+c+d}, \tag{2c}$$

$$\mathcal{L} = \frac{d}{a+b+c+d} = 1 - \mathcal{U} - \mathcal{I} - \mathcal{G}, \tag{2d}$$

where $\mathcal{U}$, $\mathcal{I}$, $\mathcal{G}$ and $\mathcal{L}$ reflect the extent of Union, Intersection, Gain and Loss models, respectively. Briefly, $\mathcal{U}$ is the fraction of microbial taxa found on hybrids and on one (but not both) progenitor (note that this is a slight deviation from the conceptual Union Model, which does not distinguish between taxa found on one or both progenitors. This deviation is necessary to avoid double counting microbial taxa in the 4H index. Also note that $\mathcal{U}$ can be divided into a component that the hybrid shares with the first progenitor, $\mathcal{U}_1$, and a component that the hybrid shares with the second progenitor, $\mathcal{U}_2$). $\mathcal{I}$ is the fraction of microbial taxa found on hybrids and on both progenitors, $\mathcal{G}$ is the fraction of microbial taxa only found on hybrids, and $\mathcal{L}$ is the fraction of microbial taxa only found on progenitors.

Similarly, for the Sorensen-inspired method, we define the four dimensions of the 4H index (three independent dimensions) as:

$$\mathcal{U} = \frac{2b}{3a+2b+c+d_1+d_2+2d_{12}}, \tag{3a}$$

$$\mathcal{U}_1 = \frac{2b_1}{3a+2b+c+d_1+d_2+2d_{12}},$$

$$\mathcal{U}_2 = \frac{2b_2}{3a+2b+c+d_1+d_2+2d_{12}},$$

$$\mathcal{I} = \frac{3a}{3a+2b+c+d_1+d_2+2d_{12}}, \tag{3b}$$

$$\mathcal{G} = \frac{c}{3a+2b+c+d_1+d_2+2d_{12}}, \tag{3c}$$

$$\mathcal{L} = \frac{d_1+d_2+2d_{12}}{3a+2b+c+d_1+d_2+2d_{12}} = 1 - \mathcal{U} - \mathcal{I} - \mathcal{G}. \tag{3d}$$

Notice that the difference between the Jaccard- and Sorensen-inspired methods is whether taxa found on multiple host classes are weighted according to the number of host classes that they occur on. Similar to the Jaccard index for beta diversity, the Jaccard-inspired 4H index only counts the unique microbial taxa shared by each combination of host classes. By contrast, like the Sorensen index for beta diversity, the Sorensen-inspired 4H index triple weights microbial taxa shared by all three host classes and double weights microbial taxa shared by two of the three host classes.

Function FourHbootstrap in HybridMicrobiomes takes a phyloseq object (McMurdie & Holmes, 2013) and a vector specifying progenitor and hybrid classifications. It then calculates the 4H index over bootstrapped samples of hybrid organisms and their progenitors. FourHbootstrap outputs a data frame with the percentage of microbial taxa that fall into each of the four models (see Equation 1). The data frame also includes the fraction of progenitor microbial taxa that are found on both progenitors. Finally, the data frame breaks the Union Model into separate components attributable to the first progenitor and the second progenitor, respectively ($\mathcal{U}_1 + \mathcal{U}_2 = \mathcal{U}$).

2.2.2 | Abundance-based analyses

Like incidence, we include two different abundance-based methods for calculating the 4H index, with the first inspired by the Ruzicka index (Legendre, 2014) and the second inspired by the Bray–Curtis index (Bray & Curtis, 1957). Similar to incidence-based methods, the two abundance-based methods also focus on core microbial taxa as defined by ρ, ϑ and ε. However, abundance-based methods require an additional pre-step to find representative microbial abundances for each host class. This step is performed on the full microbiome (i.e. core and non-core microbial taxa) based on either the mean or median relative abundance of each microbial taxon on each host class. These representative abundances can then be used raw or can be renormalized based only on microbial taxa that comprise the core of each host class. Renormalization results in a metric that is density invariant (i.e. does not vary with the number of reads attributed to the core microbiome of each host class) (Jost et al., 2011). However, a downside of renormalization is that it constrains the 4H index to a two-dimensional plane (the same is true when using $\rho = 0$ since the FourHbootstrapA function rarefies microbiome data sets, thereby forcing all hybrid classes to have equivalent numbers of reads). By contrast, using raw reads allows the total number of reads to differ between host classes. While this results in a metric that is not density invariant (i.e. it changes with the number of reads attributed to the core of a particular host class, see Supplemental Information S1), we view raw reads as the preferred option. This is because full microbiomes are rarefied to the same number of reads prior to selection

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of the core. Thus, differences in read numbers attributed to the core reflect biologically meaningful differences in composition. To generalize our incidence-based 4H index to account for abundance, we follow the method in Tamás et al. (2001) and define:

$$A = \sum_{i=1}^{S} \min\left(\min\left(x_{iP_1}, x_{iP_2}\right), x_{iH}\right) = \sum_{i=1}^{S} \alpha_i. \tag{4a}$$

$$B = \sum_{i=1}^{S} \left\{\min\left(x_{iP_1} - \alpha_i, x_{iH} - \alpha_i\right) + \min\left(x_{iP_2} - \alpha_i, x_{iH} - \alpha_i\right)\right\} = \sum_{i=1}^{S} \left\{\beta_{1i} + \beta_{2i}\right\}, \tag{4b}$$

$$B_1 = \sum_{i=1}^{S} \left\{\min\left(x_{iP_1} - \alpha_i, x_{iH} - \alpha_i\right)\right\} = \sum_{i=1}^{S} \beta_{1i},$$

$$B_2 = \sum_{i=1}^{S} \left\{\min\left(x_{iP_2} - \alpha_i, x_{iH} - \alpha_i\right)\right\} = \sum_{i=1}^{S} \beta_{2i},$$

$$C = \sum_{i=1}^{S} \left\{x_{iH} - \alpha_i - \beta_{1i} - \beta_{2i}\right\}, \tag{4c}$$

$$D = \sum_{i=1}^{S} \left\{x_{iP_1} + x_{iP_2} - \alpha_i - \beta_{1i} - \beta_{2i} - \min\left(x_{iP_1}, x_{iP_2}\right)\right\}, \tag{4d}$$

$$D_1 = \sum_{i=1}^{S} \left\{x_{iP_1} - \beta_{1i} - \min\left(x_{iP_1}, x_{iP_2}\right)\right\},$$

$$D_2 = \sum_{i=1}^{S} \left\{x_{iP_2} - \beta_{2i} - \min\left(x_{iP_1}, x_{iP_2}\right)\right\},$$

$$D_{12} = \sum_{i=1}^{S} \left\{\min\left(x_{iP_1}, x_{iP_2}\right) - \alpha_i\right\},$$

where x_{iP_1}, x_{iP_2} and x_{iH} are the number/fraction of reads of microbial taxon i on the first progenitor, the second progenitor and the hybrid respectively, and S is the total number of microbial taxa in the system. A is then the number/fraction of reads shared by both progenitors and the hybrid, B is the number/fraction of reads shared by one progenitor (but not both) and the hybrid, B_1 is the number/fraction of reads shared by the first progenitor and the hybrid, B_2 is the number/fraction of reads shared by the second progenitor and the hybrid, C is the number/fraction of reads found only on the hybrid, D is the number/fraction of reads found only on one or both progenitors, D_1 is the number/fraction of reads found only on the first progenitor, D_2 is the number/fraction of reads found only on the second progenitor and D_{12} is the number/fraction of reads found only on both progenitors. For the Ruzicka-inspired method, we define the four dimensions of the 4H index (three independent dimensions) as:

$$\mathcal{U} = \frac{B}{A+B+C+D}, \tag{5a}$$

$$\mathcal{U}_1 = \frac{B_1}{A+B+C+D},$$

$$\mathcal{U}_2 = \frac{B_2}{A+B+C+D},$$

$$\mathcal{I} = \frac{A}{A+B+C+D}, \tag{5b}$$

$$\mathcal{G} = \frac{C}{A+B+C+D}, \tag{5c}$$

$$\mathcal{L} = \frac{D}{A+B+C+D} = 1 - \mathcal{U} - \mathcal{I} - \mathcal{G}. \tag{5d}$$

Similarly, for the Bray–Curtis-inspired method, we define the four dimensions of the 4H index (three independent dimensions) as:

$$\mathcal{U} = \frac{2B}{3A+2B+C+D_1+D_2+2D_{12}}, \tag{6a}$$

$$\mathcal{U}_1 = \frac{2B_1}{3A+2B+C+D_1+D_2+2D_{12}},$$

$$\mathcal{U}_2 = \frac{2B_2}{3A+2B+C+D_1+D_2+2D_{12}},$$

$$\mathcal{I} = \frac{3A}{3A+2B+C+D_1+D_2+2D_{12}}, \tag{6b}$$

$$\mathcal{G} = \frac{C}{3A+2B+C+D_1+D_2+2D_{12}}, \tag{6c}$$

$$\mathcal{L} = \frac{D_1+D_2+2D_{12}}{3A+2B+C+D_1+D_2+2D_{12}} = 1 - \mathcal{U} - \mathcal{I} - \mathcal{G}. \tag{6d}$$

Again, the difference between the Ruzicka- and Bray–Curtis-inspired methods is whether reads/fractions of reads found on multiple host classes are weighted according to the number of host classes that they occur on. Similar to the Ruzicka index for beta diversity, the Ruzicka-inspired 4H index only counts shared microbial reads once regardless of the number of host classes that they occur on. By contrast, like the Bray–Curtis index for beta diversity, the Bray–Curtis-inspired 4H index triple weights reads/fractions of reads shared by all three host classes and double weights reads/fractions of reads shared by two of the three host classes. Function FourHbootstrapA in HybridMicrobiomes performs abundance-based bootstraps of the 4H index with input and output as described for the function FourHbootstrap (see above).

2.2.3 | Bootstrap analysis

Function FourHcentroid takes the output from FourHbootstrap or FourHbootstrapA and calculates the centroid of the bootstrapped samples. Function FourHcompare takes the outputs from FourHbootstrap or FourHbootstrapA on multiple hybrid systems and uses a PERMANOVA test (Anderson, 2014) on the isometric log-ratio transformed (Egozcue et al., 2003) 4H indices (with the option to use a centred log-ratio transformation, an additive log-ratio transformation or untransformed data instead (Filzmoser et al., 2010; Quinn et al., 2019)) to determine whether different hybrid systems vary with respect to the importance of the Union, Intersection, Gain and Loss models, respectively.

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2.2.4 | Quaternary plots

To visualize the 4H index (see Figures 2 and 3), which can be particularly helpful for comparison between systems, we introduce a quaternary plotting technique (i.e. a four-dimensional barycentric plot or an Aitchison Simplex (Aitchison, 1982)). This positions each of our four index dimensions ($\mathcal{U}$, $\mathcal{I}$, $\mathcal{G}$ and $\mathcal{L}$) at a vertex of a triangular prism, with one edge of the prism connecting the Gain and Loss models (henceforth termed the 'transgressive axis') and the opposite edge connecting the Union and Intersection models (henceforth termed the 'parental axis'). Function FourHquaternary takes the output from FourHbootstrap or FourHbootstrapA and generates an interactive and rotatable quaternary plot of the bootstrapped samples with the option to include the centroid. Function FourHquaternarycentroid takes the output from FourHbootstrap or FourHbootstrapA and generates an interactive quaternary plot of only the centroids over the bootstrapped samples.

2.2.5 | Null planes

As suggested above (see 'The Loss Model'), both our conceptual models and the four dimensions of the 4H index conflate microbial taxon loss due to the intersection of progenitor microbiomes (i.e. loss of microbial taxa only present on one progenitor) with broader microbial taxon loss (i.e. including loss of microbial taxa present on both progenitors). Thus, the 4H index does not indicate whether the microbial taxa that are lost versus retained by hybrid organisms represent microbial taxa that are shared by both progenitors or taxa that are only found on one progenitor. Unfortunately, conflation of these different types of loss is necessary to double-counting microbial taxa ($\mathcal{U} + \mathcal{I} + \mathcal{G} + \mathcal{L} = 1$) while still using a maximum of four (beneficial for visualization) dimensions. To offset this constraint, and better identify the particular microbial taxa that are lost by hybrid organisms, we develop 'null planes'. Specifically, we assume a null model wherein all microbial taxa (or reads in the case of abundance-based methods) present on progenitors are equally likely to be lost

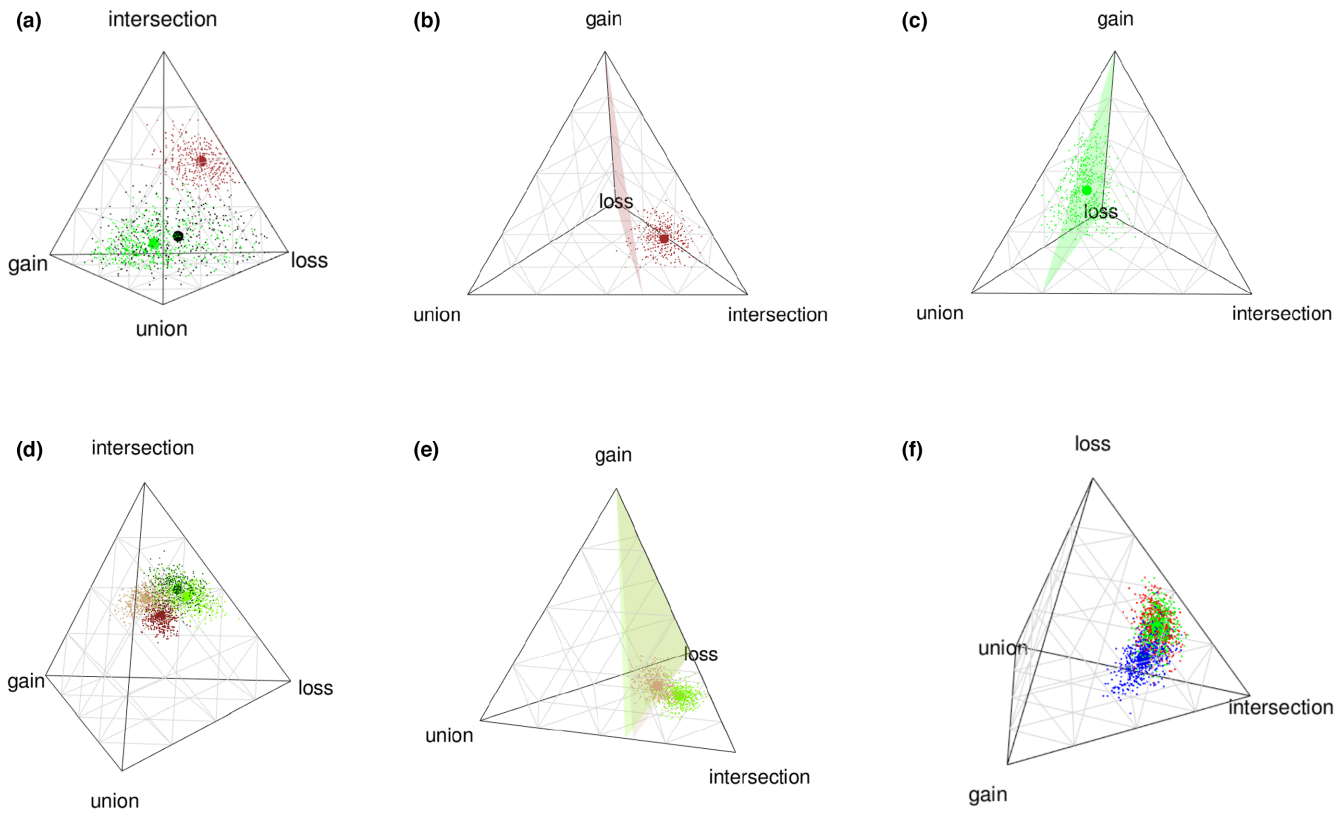

FIGURE 2 Quaternary plots showing 500 bootstrapped genus-level microbial samples (small circles) and the bootstrap centroid (large circles) of the Jaccard-inspired 4H index for (a) gut microbiomes from hybrid *Kikihia* cicadas (black), *Neotoma* woodrats (brown) and *Aspidoscelis neomexicanus* whiptail lizards (green); (b, c) woodrat and lizard systems individually along with the system null planes; (d) leaf (green) and rhizosphere (brown) bacterial/archaeal (16S rRNA, light) and fungal (ITS, dark) microbiomes from B73 line × Mo17 line maize hybrids; (e) B73 line × Mo17 line maize hybrid leaf and rhizosphere bacterial/archaeal systems along with system null planes; (f) leaf bacterial/archaeal microbiomes from B73 line × Mo17 line (red), B73 line × CML103 line (green) and B73 line ×Mo18W line (blue) maize hybrids. For systems in (a–c), bootstraps consisted of seven hybrid individuals and seven of each progenitor. For systems in (d–f), bootstraps consisted of 10 hybrid individuals and 10 of each progenitor. A microbial genus was defined as being part of the core microbiome if at least 50% of hosts from a particular class carried that microbial genus.

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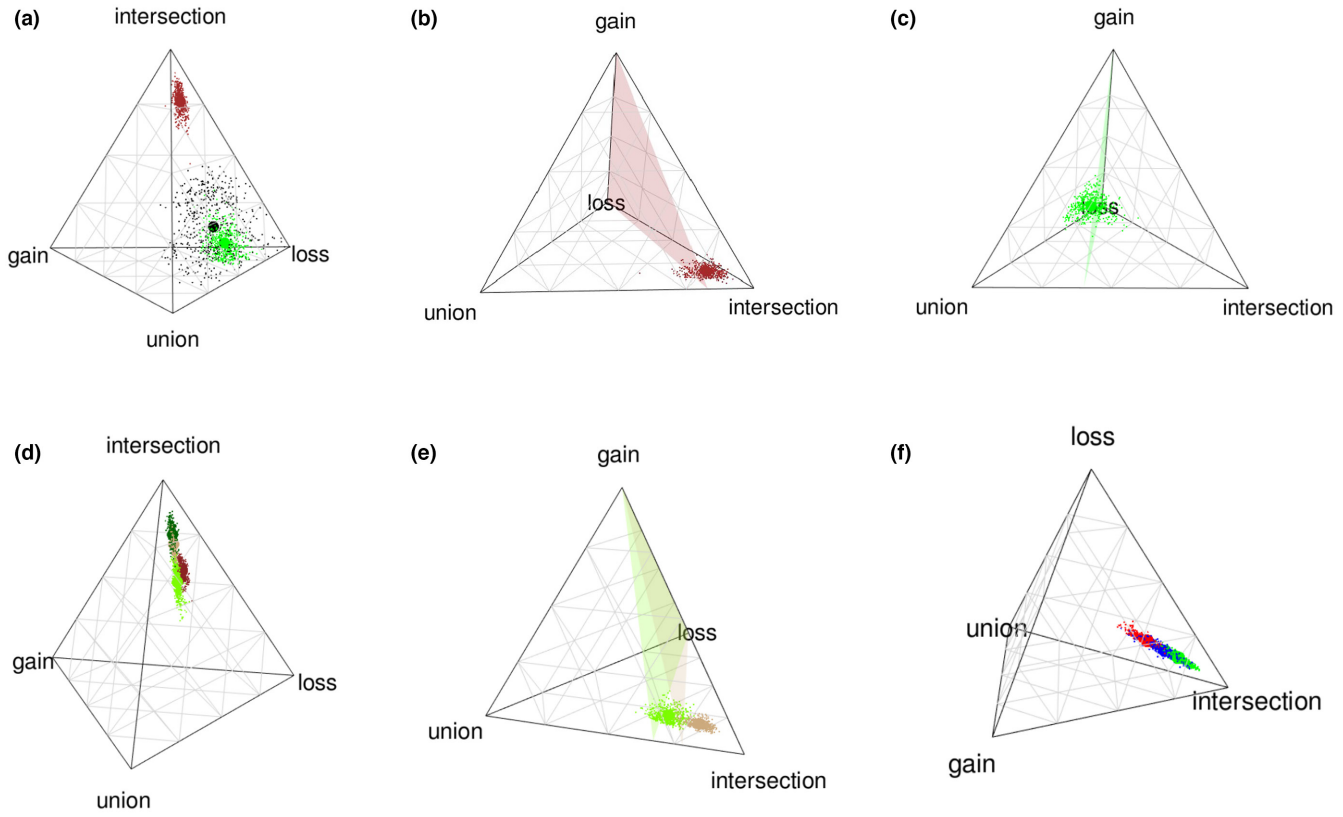

FIGURE 3 Quaternary plots showing 500 bootstrapped genus-level microbial samples (small circles) and the bootstrap centroid (large circles) of the Bray–Curtis-inspired 4H index for (a) gut microbiomes from hybrid *Kikihia* cicadas (black), *Neotoma* woodrats (brown) and *Aspidoscelis neomexicanus* whiptail lizards (green); (b, c) woodrat and lizard systems individually along with the system null planes; (d) leaf (green) and rhizosphere (brown) bacterial/archaeal (16S rRNA, light) and fungal (ITS, dark) microbiomes from B73 line × Mo17 line maize hybrids; (e) B73 line × Mo17 line maize hybrid leaf and rhizosphere bacterial/archaeal systems along with system null planes; (f) leaf bacterial/archaeal microbiomes from B73 line × Mo17 line (red), B73 line × CML103 line (green) and B73 line ×Mo18W line (blue) maize hybrids. For systems in (a–c), bootstraps consisted of seven hybrid individuals and seven of each progenitor. For systems in (d–f), bootstraps consisted of 10 hybrid individuals and 10 of each progenitor. A microbial genus was defined as being part of the core microbiome if at least 50% of hosts from a particular class carried that microbial genus.

by hybrids. This allows us to define a plane bisecting the quaternary plot at the expected fraction of hybrid microbial taxa that should be shared with one versus both progenitors, assuming that there is no preferential loss of one over the other. For any value of $\theta = \mathscr{G} + \mathscr{L}$ (i.e. the summed fractions of microbial taxa following the Gain and Loss models), the null plane is given by:

$$\mathscr{U}_{\text{null}} = (1-\sigma)(1-\theta), \tag{7a}$$

$$\mathscr{I}_{\text{null}} = \sigma(1-\theta), \tag{7b}$$

where $\sigma = \frac{a+d_{12}}{a+d_{12}+d_1+d_2}$ for the Jaccard-inspired 4H index, $\sigma = \frac{2(a+d_{12})}{2(a+d_{12})+d_1+d_2}$ for the Sorensen-inspired 4H index, $\sigma = \frac{A+D_{12}}{A+D_{12}+D_1+D_2}$ for the Ruzicka-inspired 4H index and $\sigma = \frac{2(A+D_{12})}{2(A+D_{12})+D_1+D_2}$ for the Bray–Curtis-inspired 4H index. In general, σ is the fraction of parental microbial taxa that are found on both progenitors. $\mathscr{U}_{\text{null}}$ and $\mathscr{I}_{\text{null}}$ are thus the expected fractions of microbial taxa that should be found on only one parent versus both parents under null model assumptions. 4H indices that lie more towards the $\mathscr{I}$ vertex relative to the null plane indicate that hybrids are disproportionately likely to retain microbes shared by both progenitors (i.e. loss is concentrated among microbial taxa only found on one of the two progenitors as in the Intersection Model). 4H indices that lie more towards the $\mathscr{U}$ vertex relative to the null plane suggest that hybrids are disproportionately likely to retain microbes only found on one of the two progenitors (i.e. loss is concentrated among microbial taxa found on both progenitors and is saltational). By using the null plane as a reference, it is possible to assess the degree to which the loss occurs due to the intersection of progenitor microbiomes versus the broader 'saltational' loss of microbes present on both progenitors. Function FourHnullplane takes the output from FourHbootstrap or FourHbootstrapA and graphs the (average) null plane for a particular hybrid system onto a quaternary plot. Function FourHplaneD takes the output from FourHbootstrap or FourHbootstrapA and reports both the average distance between the expected, $\mathscr{I}_{\text{null}}$, and observed, $\mathscr{I}$, value of the intersection dimension, as well as the fraction, *p*, of bootstrap samples that lie further from the $\mathscr{I}$ vertex than expected (this is useful for testing the hypothesis that microbes shared by both progenitors are more likely to be retained by the hybrid than microbes only found on one progenitor).

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3 | RESULTS

To illustrate the usefulness of the 4H index, we apply functions from our HybridMicrobiomes package to a range of plant and animal hybrid microbiota data sets including results from both 16S rRNA gene and ITS sequencing (see Supplemental Information S2 for additional system pre-analysis steps and Supplemental Information S3 for more information on the organismal systems). These analyses demonstrate how any given hybrid system shows mixed support for each of our conceptual models and how the degree of support for any particular conceptual model varies from one system to another. First, we consider applying the Jaccard-inspired 4H index (incidence-based) to our data sets (see Figure 2; Tables 1 and 2; Supplemental Video files Figures 2a.mp4–2f.mp4). Figure 2a shows quaternary plots (R version 4.2.1, phyloseq 1.41.1) comparing gut microbiota from F_1 crosses of *Neotoma* woodrats (brown) (Nielsen et al., 2023), *Kikihia* cicadas with evidence of mitochondrial introgression (black) (Haji et al., 2022) and a parthenogenetic *Aspidoscelis* lizard of hybrid origin (green, our own data). Table 1 shows the average values of the 4H indices for each system. Table 2 shows the average values of σ (the fraction of the overall parental microbiome found on both progenitors), the mean distance between the predicted and observed value of $\mathscr{I}$ and the proportion of bootstrap samples that lie further from the $\mathscr{I}$ vertex than expected based on the null model. Despite the variation in life history (vertebrate vs. invertebrate, ectotherm vs. endotherm, herbivore vs. insectivore) and mode of hybridization (F_1 crosses, mitochondrial introgression, hybrid speciation/parthenogenesis), the 4H index enables comparison across all systems. Hybrid woodrats, for example, are dominated by the Intersection Model ($\mathscr{I} = 0.4927$), while this is less important for hybrid cicadas ($\mathscr{I} = 0.1337$) and hybrid lizards ($\mathscr{I} = 0.1160$). Instead, lizards and cicadas feature a mix of the Gain and Loss models, with Gain being more important for lizards ($\mathscr{G} = 0.3438$) and Loss being more important for cicadas ($\mathscr{L} = 0.3758$). Figure 2b,c illustrates null planes for the woodrat and lizard systems. From the null planes and Table 2, we see that the two progenitor woodrat species share a larger fraction of their core microbes as compared to the two progenitor lizard species. Furthermore, we see that hybrid woodrats are biased towards the Intersection Model as compared to the null plane ($\mathscr{I} - \mathscr{I}_{null} = 0.1358$, $p = 0.002$). This means that hybrid woodrats are more likely to retain microbial taxa that are shared by both progenitors than they are to retain microbial taxa found on only one of the two progenitors. By contrast, hybrid lizards do not show any obvious bias, and thus, are equally likely to retain (or lose) microbial taxa that are shared by both progenitors or only present on one of the two progenitors.

Figure 2d shows quaternary plots of both the phyllosphere (green) and rhizosphere (brown) of hybrid maize (B73 line × Mo17 line) for both bacterial/archaeal (16S rRNA gene, light shade) and fungal (ITS1 gene, dark shade) microbiotas (Wagner et al., 2020). Figure 2e shows the same bacterial/archaeal microbiotas but includes their respective null planes, and Figure 2f compares the bacterial/archaeal phyllosphere microbiotas across three different maize hybrids: B73 line × Mo17 line (red; stiff stalk crossed with non-stiff stalk varieties (Wagner et al., 2020)), B73 line × CML103 line (yellow; temperate crossed with tropical varieties (Woodhouse

TABLE 1 Centroid values of the Jaccard-inspired 4H index as calculated by the FourHcentroid function.

	Parental axis			Transgressive axis		
	$\mathscr{U}$	$\mathscr{I}$	$\mathscr{U} + \mathscr{I}$	$\mathscr{G}$	$\mathscr{L}$	$\mathscr{G} + \mathscr{L}$
Neotoma woodrat	0.07296	0.4927	0.5657	0.0811	0.3532	0.4343
Aspidoscelis lizard	0.2941	0.1160	0.4101	0.3438	0.2461	0.5899
Kikihia cicada	0.1999	0.1337	0.3336	0.2906	0.3758	0.6664
Maize B73 × Mo17 (leaf, bacteria)	0.1272	0.4949	0.6221	0.0575	0.3205	0.3780
Maize B73 × Mo17 (rhizosphere, bacteria)	0.1892	0.5000	0.6892	0.1624	0.1484	0.3108
Maize B73 × Mo17 (leaf, fungi)	0.1029	0.5238	0.6267	0.0902	0.2832	0.3734
Maize B73 × Mo17 (rhizosphere, fungi)	0.1936	0.4226	0.6162	0.1524	0.2314	0.3838
Maize B73 × CML103 (leaf, bacteria)	0.1070	0.5044	0.6114	0.0798	0.3087	0.3885
Maize B73 × Mo18W (leaf, bacteria)	0.1601	0.4784	0.6385	0.1632	0.1983	0.3615
Maize B73 × CML103 (rhizosphere, fungi)	0.2152	0.4468	0.6620	0.1123	0.2256	0.3379
Maize B73 × Mo18W (rhizosphere, fungi)	0.1433	0.4635	0.6068	0.1146	0.2785	0.3931

Note: Summing the values $\mathscr{U} + \mathscr{I}$ and $\mathscr{G} + \mathscr{L}$ gives totals along the parental axis and the transgressive axis, respectively, and can be used as a broader scale comparison between systems.

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TABLE 2 Fraction of shared microbial taxa among progenitors, σ, as calculated by the FourHcentroid function for the Jaccard-inspired 4H index. Displacement from the null plane ($\mathscr{I} - \mathscr{I}_{null}$)[a] and the proportion of bootstrap samples (p) falling further from the $\mathscr{I}$ vertex than the null plane are both calculated by the FourHnullplaneD function.

	σ	$\mathscr{I} - \mathscr{I}_{null}$	p
Neotoma woodrat	0.6273	0.1358	0.002
Aspidoscelis lizard	0.2490	0.0110	0.364
Kikihia cicada	0.2827	0.0373	0.178
Maize B73×Mo17 (leaf, bacteria)	0.5984	0.1208	0
Maize B73×Mo17 (rhizosphere, bacteria)	0.6291	0.0663	0
Maize B73×Mo17 (leaf, fungi)	0.6483	0.1167	0
Maize B73×Mo17 (rhizosphere, fungi)	0.5664	0.0742	0
Maize B73×CML103 (leaf, bacteria)	0.6475	0.1077	0
Maize B73×Mo18W (leaf, bacteria)	0.6121	0.0872	0
Maize B73×CML103 (rhizosphere, fungi)	0.5565	0.0781	0
Maize B73×Mo18W (rhizosphere, fungi)	0.6145	0.0910	0

[a]Positive values of $\mathscr{I} - \mathscr{I}_{null}$ indicate that points lie closer to the intersection vertex than expected by chance, suggesting that hybrids are more likely to retain taxa shared by both progenitors than they are to retain taxa shared by only one of the two progenitors. Negative values indicate the opposite, namely that hybrids are more likely to retain taxa only found on one of the two progenitors than they are to retain taxa found on both progenitors.

et al., 2021)) and B73 line × Mo18W line (blue; flooding sensitive crossed with flooding insensitive varieties (Campbell et al., 2015)). Tables 1 and 2 show the corresponding values of the 4H indices, as well as relationships of the hybrid microbiotas to their respective null planes. As with our animal examples, our analysis of maize hybrids demonstrates the versatility of the 4H index and how the 4H index can be used to compare not only between microbiotas from different host species but also between microbiotas from different parts of a single organism (roots vs. leaf) or different microbial taxonomic groups (bacteria vs. fungi). Notably, the entire maize system is dominated by the Intersection Model as seen by the nearly 50% or more of model support across all comparisons (see Table 1). However, the hybrid rhizosphere (brown) is more prone to Union and Gain. This is apparent from its relatively greater clustering nearest to the Union and Gain vertices, as well as relatively greater support for these two models (see Figure 2d; Table 1; Supplemental Video files Figure 2d.mp4). By contrast, the hybrid phyllosphere is more prone to Loss. This is apparent from its relatively greater clustering near the Loss vertex and relatively greater support for the Loss Model (see Figure 2d; Table 1; Supplemental Video files Figure 2d.mp4).

Next, we consider applying the Bray–Curtis-inspired 4H index (abundance-based) to our data sets (see Figure 3; Tables 3 and 4; Supplemental Video files Figures 3a.mp4–3f.mp4). Similar to the Jaccard-inspired 4H index (see Figure 2; Table 1), hybrid woodrats are dominated by the Intersection Model ($\mathscr{I} = 0.7745$), while this is less important for hybrid cicadas ($\mathscr{I} = 0.1645$) and hybrid lizards ($\mathscr{I} = 0.07921$). Instead, lizards and cicadas feature a mix of the Gain and Loss models. For the Bray–Curtis-inspired index, however, the Gain Model is almost equivalent for lizards ($\mathscr{G} = 0.1411$) and cicadas ($\mathscr{G} = 0.1420$). Meanwhile, the Loss Model is slightly more important for lizards ($\mathscr{L} = 0.5705$) as compared to cicadas ($\mathscr{L} = 0.4993$), which is the reverse of findings for the Jaccard-inspired 4H index. Subtle differences in results based on the chosen metric are consistent with the different interpretations of the metrics. In this case, for instance, community membership differences suggest that lizard hybrids feature more Gain and less Loss, but that these differences are insignificant or reversed when considering abundance changes. Such discrepancies are expected when membership changes occur primarily in rare taxa and thus contribute little to abundance change, which may instead be dominated by shifts in abundance of microbes shared by hybrids and progenitors. From the null planes and Table 4, we see that, consistent with the Jaccard-inspired 4H index, the Bray–Curtis-inspired 4H index suggests that the two progenitor woodrat species share a larger fraction of their core microbes as compared to the two progenitor lizard species. Furthermore, hybrid woodrats are biased towards retaining microbes shared by both progenitors ($\mathscr{I} - \mathscr{I}_{null} = 0.0476518$, $p = 0.006$), whereas hybrid lizards do not preferentially retain microbes based on whether they are shared by one or both progenitors ($\mathscr{I} - \mathscr{I}_{null} = -0.030863$, $p = 0.764$).

Like our animal examples, abundance- and incidence-based 4H indices for maize hybrids exhibit a similar pattern. In particular, with our abundance-based analysis, we again find that the entire maize system is dominated by the Intersection Model. Indeed, like woodrats, the dominance of the Intersection Model is even more apparent for the Bray–Curtis-inspired 4H index than it is for the Jaccard-inspired 4H index with >60% of model support across all comparisons (see Table 3). However, differences between the hybrid rhizosphere (brown) and hybrid phyllosphere (green) are not as obvious and/or are reversed when changes in abundance are accounted for. Again, this suggests that microbiota membership changes on the hybrid are sometimes but not always consistent with abundance changes.

One of the benefits of the 4H index is the fact that it can be applied to any hybrid system, regardless of the type of host, the type of microbiome, microbiome composition or even microbiome diversity. This flexibility follows from the fact that the 4H index is monotonic with respect to each vertex/dimension ($\mathscr{U}$, $\mathscr{I}$, $\mathscr{G}$ and $\mathscr{L}$), optionally density invariant and replication invariant (see Supplemental Information S1) (Magurran & McGill, 2010). Despite this, some standardization of data sets from different systems is necessary for fair comparison. For example, the 4H index can be applied to microbiomes at any taxonomic scale. As expected, however, higher taxonomic scales predict a greater importance of the Intersection

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TABLE 3 Centroid values of the Bray–Curtis-inspired 4H index as calculated by the FourHcentroid function.

	Parental axis			Transgressive axis		
	$\mathscr{U}$	$\mathscr{I}$	$\mathscr{U}+\mathscr{I}$	$\mathscr{G}$	$\mathscr{L}$	$\mathscr{G}+\mathscr{L}$
Neotoma woodrat	0.1011	0.7745	0.8756	0.02852	0.09587	0.12439
Aspidoscelis lizard	0.2092	0.07921	0.28841	0.1411	0.5705	0.7116
Kikihia cicada	0.1942	0.1645	0.3587	0.1420	0.4993	0.6413
Maize B73×Mo17 (leaf, bacteria)	0.2326	0.5790	0.8116	0.02496	0.1635	0.1884
Maize B73×Mo17 (rhizosphere, bacteria)	0.1199	0.7311	0.8510	0.02944	0.1196	0.1490
Maize B73×Mo17 (leaf, fungi)	0.08865	0.7709	0.8596	0.03248	0.1079	0.1404
Maize B73×Mo17 (rhizosphere, fungi)	0.1157	0.6092	0.7248	0.06660	0.2086	0.2752
Maize B73×CML103 (leaf, bacteria)	0.1094	0.7372	0.8467	0.03673	0.1166	0.1533
Maize B73×Mo18W (leaf, bacteria)	0.1611	0.6719	0.8330	0.03272	0.1343	0.1670
Maize B73×CML103 (rhizosphere, fungi)	0.1542	0.6257	0.7800	0.05275	0.1673	0.2200
Maize B73×Mo18W (rhizosphere, fungi)	0.1357	0.6090	0.7447	0.06074	0.1945	0.2553

Note: Summing the values $\mathscr{U}+\mathscr{I}$ and $\mathscr{G}+\mathscr{L}$ gives totals along the parental axis and the transgressive axis, respectively, and can be used as a broader scale comparison between systems.

Model because hybrids are more likely to share distantly related microbial taxa with progenitors than they are to share identical or near-identical microbial taxa (see Figures S1.1–S1.4; Tables S1.1 and S1.2). Importantly, because taxonomic scale can have considerable effects, systems should always be compared using the same taxonomic scale, and interpretation of the index should always be within the context of the taxonomic scale chosen. Likewise, defining the core microbiome based on a lower fraction of hosts also favours Intersection (at least some hybrids and some of each parental species are likely to have a particular microbial taxon, even if it is just a transient acquisition from the environment; see Figures S2.1–S2.4; Tables S2.1 and S2.2). Again, then, it is important to use the same value of ρ for all systems that are being compared. Host sample size has a smaller, but still detectable, effect resulting in somewhat different trends across systems but generally shifting the 4H index towards the parental axis and away from the transgressive axis (see Figures S3.1–S3.4; Tables S3.1 and S3.2). Although the effect of host sample size is relatively small, particularly for larger host sample sizes, it is still best to compare systems by subsampling to the smallest number of hosts available for any host class across all systems (e.g. see Figure 2 where we were limited to 7 individuals based on the number of available cicada microbiotas). Finally, sequencing depth has almost no impact on predictions, at least for >1000 reads or more. This last feature of the 4H index is a benefit of focusing on core microbiomes since low abundance microbial taxa that are likely to be missed at low read depths are unlikely to be part of the core of any given species. For this reason, it is largely unnecessary to standardize for read depth across systems. Nevertheless, functions FourHbootstrap and FourHbootstrapA do have the option to rarefy microbiome samples to a lower read depth than the minimal number of reads of the lowest sample. This allows for standardization of read depth across systems.

4 | DISCUSSION

The advent of low-cost sequencing has greatly contributed to our understanding of the importance of both hybridization and HA microbiomes on host ecological traits and evolutionary consequences. These two fields come together in the study of HA microbiomes of hybrid organisms—a newly emerging area of research across disciplines ranging from agricultural science to ecology and conservation. In this paper, we integrate four conceptual models to develop a framework for understanding the relationship between hybrid microbiomes and the microbiomes of their progenitors. We then use these models to develop a four-dimensional (three independent dimensions) metric—the 4H index—to describe where a particular hybrid complex falls among our four models. Our 4H index borrows inspiration from beta diversity metrics, and thus takes on four different forms; two are incidence-based (Jaccard-inspired and Sorensen-inspired), and two are abundance-based (Bray–Curtis-inspired and Ruzicka-inspired). Importantly, the 4H index facilitates comparisons across widely disparate systems, ultimately making it possible to identify patterns that emerge across hybrid microbiomes from different organisms. For example, the 4H index could be used to determine whether there are systematic differences between

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TABLE 4 Fraction of shared microbial taxa among progenitors, σ, as calculated by the FourHcentroid function for the Bray–Curtis-inspired 4H index. Displacement from the null plane ($\mathcal{I} - \mathcal{I}_{null}$)[a] and the proportion of bootstrap samples (p) falling further from the $\mathcal{I}$ vertex than the null plane are both calculated by the FourHnullplaneD function.

	σ	$\mathcal{I} - \mathcal{I}_{null}$	p
Neotoma woodrat	0.830025	0.0476518	0.006
Aspidoscelis lizard	0.392932	−0.030863	0.764
Kikihia cicada	0.3025042	0.05380129	0.19
Maize B73×Mo17 (leaf, bacteria)	0.6684608	0.03651511	0.132
Maize B73×Mo17 (rhizosphere, bacteria)	0.7801631	0.06678432	0
Maize B73×Mo17 (leaf, fungi)	0.8294745	0.05757388	0
Maize B73×Mo17 (rhizosphere, fungi)	0.7474605	0.06723603	0
Maize B73×CML103 (leaf, bacteria)	0.8038933	0.05631366	0.006
Maize B73×Mo18W (leaf, bacteria)	0.7243933	0.06803136	0.016
Maize B73×CML103 (rhizosphere, fungi)	0.7003835	0.07931675	0
Maize B73×Mo18W (rhizosphere, fungi)	0.7082141	0.0815329	0

[a]Positive values of $\mathcal{I} - \mathcal{I}_{null}$ indicate that points lie closer to the intersection vertex than expected by chance, suggesting that hybrids are more likely to retain taxa shared by both progenitors than they are to retain taxa shared by only one of the two progenitors. Negative values indicate the opposite, namely that hybrids are more likely to retain taxa only found on one of the two progenitors than they are to retain taxa found on both progenitors.

hybrid plant versus hybrid animal microbiomes, or between hybrid vertebrate versus hybrid invertebrate microbiomes. Likewise, the 4H index could be used to determine how phylogenetic and/or phenotypic distances between progenitors or ploidy level impact the hybrid microbiome.

Importantly, the intent of each of the four conceptual models and, indeed, the 4H index in general is to highlight hybrid–progenitor microbiome relationships. Thus, like beta diversity, the 4H index should be taken as a measure of pattern, not process. Just as beta diversity cannot be used to explain *why* turnover differs among communities, the 4H index should not be used to discriminate among microbiome reassembly mechanisms responsible for microbiome restructuring after host hybridization. In the woodrat system, for example, the 4H index cannot be used to explain why Intersection dominates. It may be that the hybrid woodrat immune system is refractory to all microbes not found on both progenitors. Alternatively, it could be that hybrid woodrats are restricted to habitats where both progenitors overlap and the hybrid microbiome reflects microbial exposure patterns of hybrid animals. Regardless, experimental work will always be needed to understand what drives hybrid–progenitor HA microbiome relationships observed using the 4H index. For this reason, we envision the 4H index as a tool that can be used for exploring and comparing patterns and formulating hypotheses about underlying eco-evolutionary processes of microbiome restructuring after host hybridization.

While not explicitly explored in this manuscript, the 4H index could easily be extended to examine HA microbiome functional change between hybrids and their progenitors. Indeed, when applied to function, the 4H index could be useful for forming hypotheses related to hybrid ecology and/or ecological success. However, even in this context the 4H index should be interpreted as a tool for characterizing patterns of change, rather than mechanisms. This is because microbiome function and host ecology can have bidirectional impacts, and thus, it can be challenging to delineate cause and effect. As a result, though function may provide better insight into potential pattern generating mechanisms, the 4H index is not a test for causality, but rather an exploratory tool for hypothesis generation.

Outside the context of hybridization, it is worth noting that this same framework can be applied to any triplet of host species, where one of the three host species is in some way 'intermediate' to the other two. Thus, for example, a 4H index could be calculated for the microbiomes of organisms from an ecotonal habitat, and then compared to the microbiomes of organisms from the two pure habitat types on either end of the ecotone (O'Brien et al., 2022), even if it is the same host taxon across the entire zone. Likewise, a 4H index could be calculated for species (e.g. swordtail males, *Xiphophorus nigrensis*) that exhibit three discrete size classes, with one size class being intermediate to the other two (Morris et al., 1992). Similarly, a 4H index could be calculated for captive animals fed two different pure diets as compared to captive animals fed a mixed diet. In these scenarios, the interpretation of our four conceptual models would change. However, because the 4H metric is defined solely based on distributions of microbial presence/absence or abundance across non-overlapping sets of host classes, it is valid for any analysis where there is ecological, evolutionary, morphological or physiological reason to believe that one host class falls between the other two host classes.

AUTHOR CONTRIBUTIONS

Benjamin T. Camper and Sharon Bewick wrote the first draft of the manuscript. Sharon Bewick developed the code for the R package. All authors contributed substantially to the content of the manuscript.

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CONFLICT OF INTEREST STATEMENT

No conflicts of interest have been declared.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

Cleaned data for cicadas, woodrats, lizards and maize and all scripts to generate the figures and tables for this manuscript are available on GitHub (https://github.com/bewicklab/HybridMicrobiomeFramework) and Zenodo (https://zenodo.org/records/10358091) (Camper et al., 2023). The HybridMicrobiomes R package is available from CRAN (https://cran.r-project.org/web/packages/HybridMicrobiomes/index.html).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Appendix S1: Monotonicity, density invariance and replication invariance.

Appendix S2: System pre-analysis.

Appendix S3: Example datasets.

Appendix S4: Additional figures and tables.

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