Review

Speciation by symbiosis

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In the *Origin of Species*, Darwin struggled with how continuous changes within a species lead to the emergence of discrete species. Molecular analyses have since identified nuclear genes and organelles that underpin speciation. In this review, we explore the microbiota as a third genetic component that spurs species formation. We first recall Ivan Wallin's original conception from the early 20th century on the role that bacteria play in speciation. We then describe three fundamental observations that justify a prominent role for microbes in eukaryotic speciation, consolidate exemplar studies of microbe-assisted speciation and incorporate the microbiota into classic models of speciation.

Speciation and symbiosis

The fields of microbial symbiosis (see Glossary) and speciation have achieved astonishing advances during the past two decades. The universality and significance of microbial symbionts in multicellular life is now unmistakable [1,2]. Concurrently, understanding of the genetic underpinnings of how one species becomes two is maturing in a wide array of eukaryotic species [3–5]. Symbiosis and speciation are not commonly discussed together and can seem to be odd partners in their capacity to operate synergistically in nature. Indeed, microbial symbiosis is a process by which two or more distinct organisms interact as one entity, whereas speciation is the diversifying process by which one species splits into two. Yet, since the earliest hypotheses of the symbiotic nature of organelles within the eukaryotic cell [6–8], microbial symbiosis has been put forth as an engine of novelty owing to its capacity to confer new traits [9], and to augment the rate of evolution of genetically based reproductive barriers between incipient species [10,11]. In this review, we synthesize recent studies that suggest microbe-assisted reproductive isolation is widespread, and we propose how symbionts can be more formally considered in theoretical and empirical studies of reproductive speciation.

The idea of a synergistic effect of symbiosis on speciation was first introduced almost a century ago and nearly forgotten. In 1927, microbiologist Ivan E. Wallin (Figure S1 in the supplementary material online) hypothesized in his book, *Symbionticism and the Origin of Species*, that the origin of new species primarily occurred through the acquisition of bacterial endosymbionts. The hypothesis was put forth several decades before its time as the tools to sample bacterial symbiont diversity and host–microbe interactions were not yet developed (text S1 in the supplementary material online). Although Wallin's hypothesis elicited appropriate skepticism at the time, modern biology recognizes the universality of symbiosis in shaping eukaryotic life. First, advances in profiling the microbial symbionts of hosts have lowered the technical hurdles from a century ago. Second, a new outlook is developing that places microbial symbiosis as a central discipline within the reticulated set of biological sciences. Third, and most importantly, there are several cases in which either microbes are the causal factors in reproductive isolation (RI) or chromosomal 'speciation genes' evolved by interactions with microbes. The latter cases serve as a reminder that, although speciation geneticists frequently map RI traits to nuclear genes, it is not an automatic justification to rule out microbial-assisted speciation. If one were to map speciation loci to genes involved in immunity, it would strongly implicate host–microbe interactions in the underlying processes of species formation.

The goal of this review is to complement the 70 years of research that fortified nuclear genes as a crucial agent of species formation [12] with a comprehensive assessment of microbial symbiosis in eukaryotic speciation. We demonstrate that if one views the microbiome of any given species as an extension of the genome of that host, as described within the hologenome theory [13], it then becomes intuitive that symbionts can be openly incorporated into speciation models, such as the evolution of Bateson–Dobzhansky–Muller (BDM) incompatibilities. Given the technical and

Glossary

Biological Species Concept: an operational species definition in which groups of individuals that cannot interbreed with other groups when brought into contact are considered different species.
Broad-sense symbiont-induced RI: gene–gene interactions that result in a reproductive barrier as a consequence of selection on the host to accommodate a microorganism; loss or alterations of the symbiont does not have an impact on the RI but the original genetic isolation evolved in response to host adaptations to the symbiont.
Hybrid susceptibility hypothesis: hybrids have a higher pathogen load than do parental species.
Microbial symbiosis: the study of microbe–host interactions.
Microbiota: the microbial life that lives symbiotically with a host.
Narrow-sense symbiont-induced RI: direct gene–symbiont or symbiont–symbiont interactions that result in a reproductive barrier that can be ameliorated or even removed through the elimination of the symbiont from the host (i.e. curing the host with antibiotics).
Superorganism or holobiont: terms used to highlight that multicellular eukaryotes are a collection of single organisms (i.e. eukaryotic and symbiotic cells) that together have the functional organization implicit in the definition of organism.
Symbiont: an obligate or transient microorganism that forms a parasitic, mutualistic or commensal interaction with a host.
Symbionticism: a term originally used by Ivan Wallin to distinguish intracellular, microbial symbioses from the more all-encompassing process of symbiosis that occurs between any two organisms in nature.
The Large Immune Effect: a colloquial term introduced here to refer to the collection of speciation studies that suggest immune genes play a disproportionate role in rapid and adaptive evolution relative to the rest of the genome.

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experimental progress since 1927, Wallin's theory on the symbiotic origin of species is primed for a new assessment.

Three general observations

Before we consider specific cases of symbiont-assisted speciation, it is important to summarize the recent and compelling reasons for why the processes of speciation and symbiosis are intertwined. At least three major observations can be made from the standing experimental evidence. First, microbial symbionts are universal in eukaryotes. Second, hosts typically exhibit strong specificity for microbial symbionts and their functions. Third, host immune genes are rapidly evolving in response to microbial symbionts and represent a gene family frequently involved in hybrid incompatibilities (HI). These unifying principles are discussed in Box 1.

Pre-mating isolation and microbes

By adhering to the widely accepted Biological Species Concept, most biologists equate speciation with the evolution of RI [14,15]. Under this definition, RI simply refers to those mechanisms that prevent or reduce interbreeding between populations or species. In general, two forms of RI hinder gene flow: processes acting before (pre-mating) or

Box 1. Three observations that link symbiosis to speciation

Observation 1: microbial symbionts are universal in eukaryotes

Microbial symbionts are universal and comprise the major fraction of the cellular and genetic machineries in eukaryotes. For example, if one summarizes the bacterial endowment of the average human, there are ten bacterial cells for every one human cell and 100 bacterial genes for every one human gene [64–66]. Likewise, insects harbor dense prokaryotic populations in their hindguts [67]. Plant roots and leaves are also highly populated with microbes [68,69]. Terms such as 'superorganism' [70,71] or 'hologenome' [13] are used to reflect a composite view of a eukaryotic species as the sum of its genes and cells from the eukaryotic and microbial components.

Observation 2: host specificity

Most microbial entities in eukaryotes are not transient passengers randomly acquired from the environment, but instead function with specific roles in eukaryotic nutrition [72], immunity [73–75], development [76] and reproduction [11,47,77], for instance; many of these functions can only be sustained in the presence of specific host–microbe combinations [78]. In humans, for example, the existence of three host–microbial enterotypes (i.e. characteristic microbiota structure of the gut community [79]) are strongly host dependent [80,81] and, once established, are relatively stable over time [82]. Specificity between host and microbiota could be because of host diet, geography and/or phylogenetic histories [83–86]. When diet and environment are strictly controlled for, the microbial community relationships between different species of *Nasonia* parasitoid wasps reflect the phylogenetic relationships of the host species [67]. Therefore, parallel divergence between host eukaryotic genes and members of the general microbiota might be a common phenomenon (Figure I).

Observation 3: immune genes are rapidly evolving and underpin changes in the microbiota

Components of host immunity genes are in an arms race with components of the microbiota. This gene family is in a constant struggle between managing beneficial or commensal symbionts while turning on host defenses to prevent pathogenic infections. These dynamics can generate rapid coevolutionary changes between the host genes and microbes, particularly if a change in one causes selective pressure in the other. In *Drosophila*, humans and chimps, defense and immunity genes evolve more rapidly and are under more positive selection than is the rest of the genome [87–89]. The conflict and compromise between host genes and microbes can ultimately give rise to reproductive barriers. Hybridization between two host species can lead to immune-related incompatibilities that we have categorized as 'The Large Immune Effect' on HI.

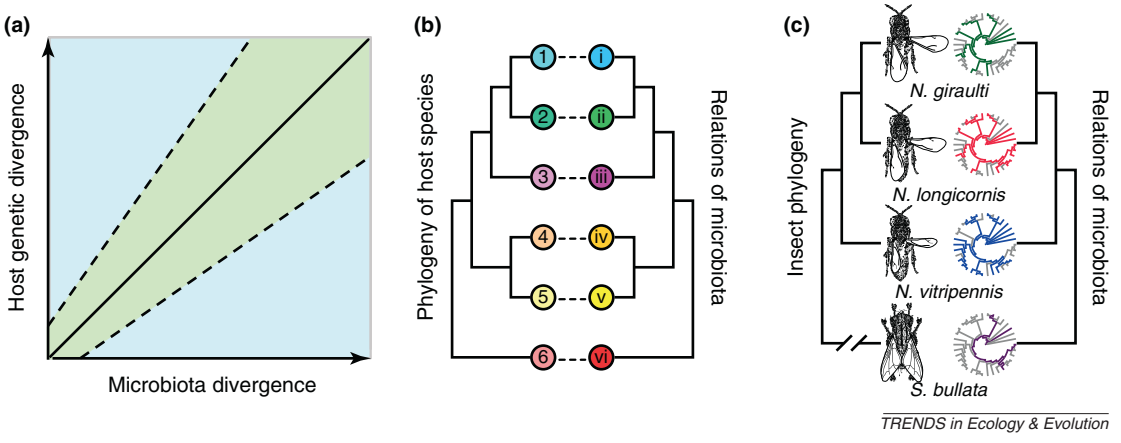

Figure I. The predicted relations between divergence in host nuclear genes, divergence in microbiotas and host speciation. **(a)** The positive relation between nuclear genetic divergence and speciation is a major tenet of genetic studies of speciation. Here, we extend this trend by theorizing that, during a host speciation event, divergence in host genes (y-axis) positively correlates with divergence in microbial communities (x-axis) and the timing of speciation (denoted by the unbroken black line). Environmental variables (the upper and lower bounds in the broken lines) can skew the correlation if they are not controlled for while sampling the host microbiota. Divergence in microbial communities can occur because of changes in both microbial species richness and/or abundance. **(b)** If the aforementioned relations operate in nature, then they can be tested in practice by comparing whether the phylogeny between host species (denoted by numbers) parallels the dendrogram of community relations between the symbiotic microbiotas (denoted by roman numerals). Note that this conceptual model assumes that, for each generation, the structure of intraspecies microbial communities will be more similar than of interspecies microbial communities and that the levels of genetic divergence between host species will associate with the relative relations of their microbial community structures. It does not presume that microbial communities are stable or even vertically transmitted from generation to generation, as endosymbionts are. **(c)** A real data example of the parallel relations between a phylogeny of insect species based on mitochondrial genes (the parasitoid species *Nasonia* and their host fly *Sarcophaga bullata*) and a dendrogram of the microbial community relations based on a bacterial gene [67]. The schematic trees are not scaled. Circular trees at the tips of the microbial community dendrogram depict the bacterial taxa present (bold) out of the total microbial community.

after (post-mating) mating. Several key studies on symbiont-assisted speciation are discussed below as well as listed in Table S1 in the supplementary material online.

Behavioral isolation

We begin with behavioral isolation because some of the most recent evidence on microbe-assisted RI comes from research on mate preferences. Behavioral isolation is a reproductive barrier that bars mating between species because of differences in courtship behavior or sexual attraction. In a striking example of symbiont-induced behavioral isolation, genetically identical *Drosophila melanogaster* that were reared on different diets (i.e. molasses and starch) acquired different microbiotas, which in turn led to strong mate discrimination between them [16]. This effect occurred after one generation of maintenance on the two diets and persisted for dozens of generations. The mate discrimination was elegantly 'cured' when the flies were treated with antibiotics. Likewise, the trait could be introduced into naïve *Drosophila* populations by inoculating them with the associated bacteria. Ultimately, the microbiota was the genetic component underlying the mating preference. In a similar experiment, mate discrimination evolved in long-term cage populations of *D. melanogaster* derived from a single maternal line and was partially cured by antibiotic treatment [17]. The putative culprit in this experiment was the widespread invertebrate symbiont, *Wolbachia*, although transfection experiments were not performed to establish a causal role of this bacterium. Recent studies also reveal that *Wolbachia* is associated with enhanced mate discrimination in *Drosophila paulistorum* [18] and reduced mate discrimination in *Nasonia* wasps [19].

At least two mechanisms could underlie these symbiont-associated mating preferences. First, the bacteria could infect the host tissues involved in mate discrimination, thereby causing a behavioral pathology that alters mate discrimination. For instance, infection of the brain or other sensory organs, as in *Nasonia* [19], could have adverse effects on how interspecific mate discrimination is regulated at the cellular and signaling levels. Second, symbiotic bacteria could either contribute to, or alter, the level of sex pheromones produced by the host. The nature by which sex pheromones are altered by microbes could be explained by either bacterial-specific molecules that act as sex attractants or by bacterial-induced effects on nuclear genes that code for sex pheromones (i.e. cuticular hydrocarbons), the latter of which has been recently demonstrated in *D. melanogaster* [20]. Furthermore, males of the grass grub beetle *Costelytra zealandica* are attracted to products generated from bacteria located in the colleterial glands, an outpocketing of the vagina of this species [21]. Additionally, associations with microorganisms can affect immunocompetence and perhaps mitigate sexual selection based on mating decisions related to immunocompetence. For instance, female mealworm beetles have a preference for males with pheromones that indicate immunocompetence [22]. Overall, microbial-induced sex attractants have been generally unexplored, but could prove significant in speciation, given the extensive distribution of normal bacterial flora of animals.

An example of a symbiont-derived courtship alteration that leads to RI in animals is observed with parthenogenesis-inducing (PI) bacteria. PI bacteria span three genera: *Wolbachia* [11], *Rickettsia* [23] and *Cardinium* [24]. They typically manipulate haplodiploid sex determination in virgin mothers, leading to the conversion of haploid eggs into diploid eggs that develop into females. Thus, all-female species can exist without any male input to reproduction.

What are the consequences of PI bacteria on species formation? The process by which an asexual population splits from a sexual population is a form of cladogenesis that can be termed 'asexual speciation'. This process falls neatly under the Biological Species Concept because it is concerned with the severing of gene flow between sexual and asexual populations [10,11]. Specifically, the onset of microbial-induced parthenogenesis does not immediately prohibit gene flow between the asexual and sexual populations because asexual females still retain the ability to mate with males from a sexual population, as in *Trichogramma* wasps [25]. However, speciation can be achieved once asexuality is established because asexual females frequently suffer from an accumulation of mutations that degenerate characters involved in sexual reproduction, including mating behaviors, secondary sexual characteristics and fertilization processes [26]. Out of six cases of reproductive degradation associated with bacterial-induced parthenogenesis, five showed female-specific degradation and the sixth exhibited both female and male sexual degradation [11]. Thus, female traits degrade more rapidly than male traits, suggesting that mutations in genes encoding female sexual traits might be selected for because they pleiotropically increase the fitness of asexual females. An alternative explanation to this pattern is that there is a substantially higher fraction of female sexual genes than male ones in the genome, which could also lead to more rapid female degradation by genetic drift. Either way, the enhancement of decay in female sexual traits strengthens the possibility for asexual speciation owing to females becoming the majority of individuals in an asexual population; the result is they will become locked into parthenogenesis. Dozens of cases of bacterial-induced parthenogenesis have been characterized within the Hymenoptera [27], suggesting that microbial-induced asexual speciation is not uncommon. However, studies on whether bacterial-induced parthenogenesis evolves before other isolation barriers are needed to rule out that speciation of the asexual lineages occurred before the evolution of bacterial-induced parthenogenesis.

Ecological isolation

Many organisms complete their entire life cycle in a single habitat, and adaptation of incipient species to different habitats is an important engine of allopatric and sympatric speciation [28]. Given enough time, it is generally accepted that bouts of positive adaptation to new habitats will drive speciation, and comparative analyses across taxa support this theory [29]. Genetic analyses of ecological isolation have focused largely on the nuclear basis of habitat specificity [30–32]. However, there is extensive evidence that, in addition to nuclear genes, bacterial symbionts play a crucial role in resource exploitation and specificity [33–35]. In fact, the use of new niches is one of the very hallmarks

that intrigued Paul Buchner, a pioneering authority on bacteriome-associated symbionts of insects [36]. He estimated that approximately 10% of insect species harbor vertically transmitted nutritional mutualists and, along with the genes of their hosts, these microbes extend the heritable genetic variation present in one species. Notably, symbiont genomes encode pathways for amino acid and vitamin synthesis that fit closely with the expected nutritional needs of their hosts [35].

Much of the evolutionary success of arthropods is attributable to the fact that they harbor endosymbionts that permit the use of a wide array of nutrient-deficient or imbalanced habitats. For instance, in the pea aphid *Acyrthosiphon pisum*, a symbiont from the gamma-proteobacteria class confers an increase in fitness on white clover plants (*Trifolium repens*) in comparison to aphids that lack this symbiont [37]. This adaptation could confer a niche expansion that leads to geographic isolation between an aphid population that makes use of white clover and an allopatric aphid population that utilizes other plants, such as vetch. In weevils, the genus *Sitophilus* is ecologically isolated from its closest relatives because its symbiotic bacteria are enclosed in a specialized structure, called a bacteriocyte, enabling it to be the only member of the Rhynochophorinae subfamily to feed exclusively on cereal grains as opposed to the roots and stems of monocotyledons [38]. The nutritional endosymbiont *Buchnera* is functionally important to more than 4400 aphid species across different plants in sympatry or allopatry [39,40]. Furthermore, variation in plants that aphids use with the aid of their *Buchnera* symbionts is tightly correlated with instances of aphid speciation [41,42]. Finally, within *A. pisum* aphids, populations exhibit variation in their amino acid requirements [34], suggesting coevolution between the amino acid supply of the symbiont–aphid combination and the amino acid deficiency of their plant diet. The aphid fossil record implies an approximate minimal date of 100–200 million years for the original infection [34] and codiversification ever since. Similar results of ancient infection and codiversification have been reported for many other bacteriome-associated symbionts, as reviewed in [43]. Codiversification patterns are not necessarily an indicator of symbiont-induced speciation, as functional work on RI must be coupled with these studies. However, if it were not for the origin of these ancient host–microbe mutualisms, major groups of arthropods would simply not exist. In the aforementioned cases, the symbionts are transmitted maternally and have been closely associated with host cells for long periods of time. Extracellular bacteria that are passed from one generation to the next can also exhibit host–symbiont cospeciation, as observed in the family Plataspidae [44].

In most of the examples mentioned above, the microbial symbiont supplements the host genome with functional genes that open up nutritional opportunities that would otherwise not be available to the host. Nutritional symbiont adaptations can confer ecological isolation between host races or species that have the symbiont and those that lack it. Any disruption of these nutritional symbioses through hybridization with other species could lead to a breakdown in genomic complementarity between the genes of the animals and the symbiont genomes and, ultimately, hybrid inferiority.

Post-mating isolation and microbes

Recent studies identifying genes involved in post-mating isolation, such as hybrid sterility and inviability, indicate that these genes can sometimes spread within populations as a consequence of genetic conflict [3,5]; these genes then cause epistatic interactions in hybrids (i.e. BDM incompatibilities; Box 2, Figure Ia,b). How do microbes fit into a standard model of post-mating isolation? The BDM model postulates that when two populations of a species evolve in isolation from each other, at least two genetic changes between the species must occur to cause negative, epistatic interactions and, thus, incompatibility in hybrids [12,14]. These HI genes evolve as a by-product of selection and/or genetic drift [12,45]. Once established between species, HI is usually irreversible.

A simple, microbial extension of the BDM model is to replace one of the nuclear genes with that of a microorganism (Box 2, Figure Ic). In other words, how many more HI can evolve when comparing a model of two nuclear loci and a symbiont (Box 2, Figure Ic) versus a model of three nuclear loci (Box 2, Figure Ib). Effectively, the number of loci is equal in both cases, three. However, this schema shows that the number of potential negative epistatic interactions underlying HI is higher in the symbiont model because a HI could now arise from an expanded network of incompatible gene–gene, gene–microbe, or microbe–microbe interactions, whereas the standard nuclear BDM model only generates gene–gene interactions. Within each of the following sections, we describe the various cases of microorganisms that cause post-mating isolation.

Cytoplasmic incompatibility

Cytoplasmic incompatibility (CI) is a post-mating incompatibility that typically leads to F_1 inviability between infected males and uninfected females, or females harboring a strain of bacteria different than that of the male. When we last reviewed the topic, *Wolbachia*-induced CI was the only phenomenon known to prevent host gene flow by a microbe–microbe interaction [11]. Expanding research in other invertebrate–microbe associations over the past decade demonstrates that *Cardinium* symbionts from the unrelated *Cytophaga–Flavobacterium–Bacteroides* phylum can also cause CI [46,47]. There have also been several important experimental and theoretical advancements that continue to support various roles for *Wolbachia*-induced CI in arthropod speciation (Box 3). In addition to bacterial-induced CI, cytoplasmic organelles of bacterial origin (i.e. mitochondria and chloroplasts) are capable of causing hybrid maladies through epistatic interactions with genes of the host nucleus.

Hybrid susceptibility

Hybridization in plants and animals can result in either: (i) genetic novelty by joining new combinations of genes from different species; or (ii) breakdown of co-adapted gene complexes causing reduced fitness, such as sterility or inviability, as reviewed in [48,49]. The outcome of hybridization is determined by many factors. As the immune system is subject to frequent bouts of positive selection and rapid evolution to combat a pathogenic microbiota and maintain a beneficial one, hybridization could spur

Box 2. The effect of symbionts on the number of hybrid incompatibilities

Following Orr and Turelli [90], population genetic theory shows that when k number of nuclear substitutions is fixed between two populations, there are (Equation I):

$$\left(\frac{k}{2}\right) = \frac{k(k-1)}{2} \quad [I]$$

possible HI. We extrapolate this model by showing that when $k = 2$, and thus there are two nuclear loci (a single nuclear HI), but the number of symbionts is allowed to increase, then the number of possible incompatibilities is (Equation II)

$$\left(\frac{s_{k=2}}{2}\right) = \frac{s(s+1)}{2} \quad [II]$$

where s is equal to the two nuclear loci plus the number of symbionts being considered. In other words, in the simplest case of when k and s equal three, and there are three nuclear loci versus two nuclear loci plus a symbiont, respectively, the model with symbionts produces more incompatibilities than the nuclear model (Figure I).

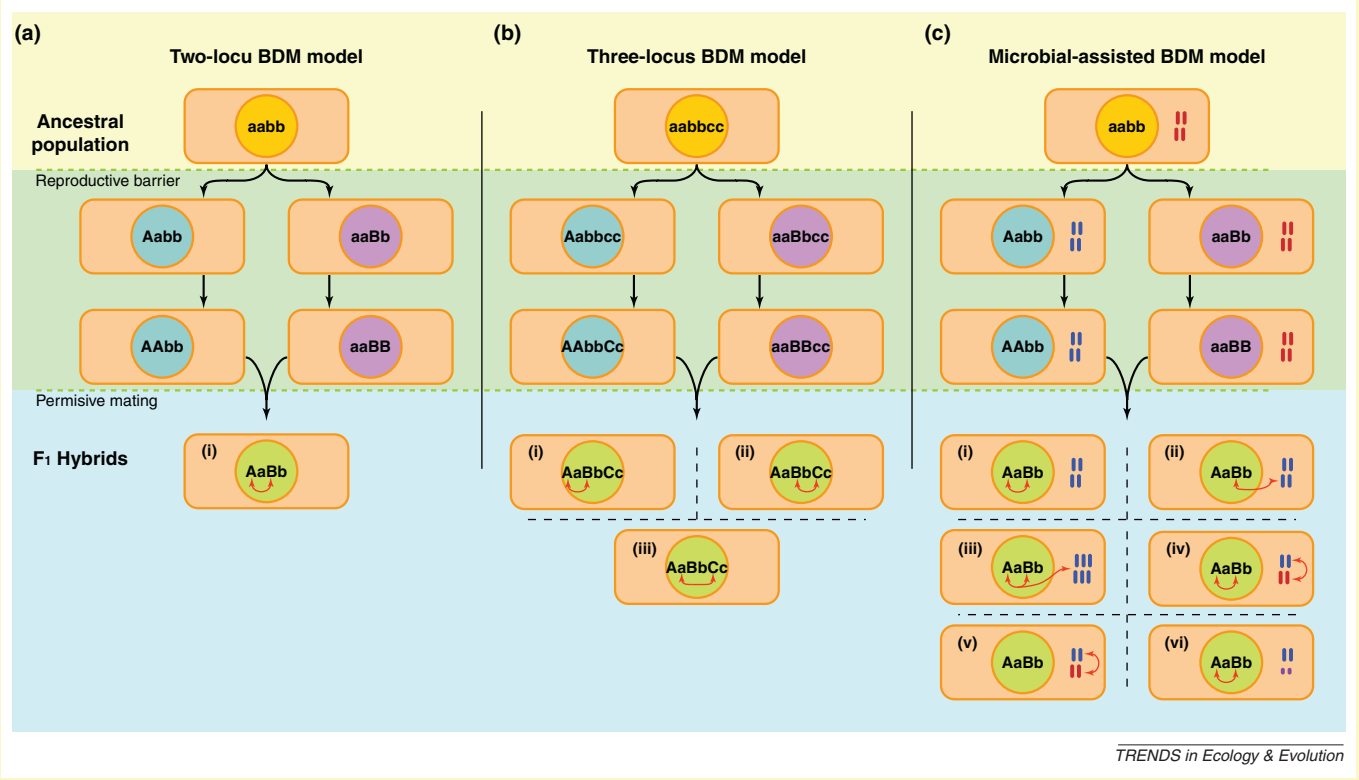

Figure I. Bateson–Dobzhansky–Muller (BDM) Models with and without microbes. A classic, two-locus BDM model of speciation **(a)** arises when substitutions in locus 'a' and locus 'b' negatively interact in hybrids (red arrows indicate potential negative epistasis) to cause one hybrid incompatibility (HI). In a three-locus BDM model of speciation **(b)**, the number of negative epistatic interactions increases from one to three. By extending these models, one can ask, when a bacterial symbiont replaces the third nuclear loci, how many new interactions can evolve? **(c)** Divergence in the two nuclear loci and a single symbiont produces six possible incompatibilities, twice as many as when there are three nuclear loci. These six HIs include: **(i)** a typical BDM that does not impact the microbial community; **(ii)** a single derived allele from one population negatively interacting with the derived bacterial symbiont of the other population; **(iii)** a three-locus interaction between the two derived mutations and the derived symbiont genotype; **(iv)** a microbe–microbe interaction between two symbiont genotypes that occurs only when there is also a negative interaction between the two derived nuclear alleles; **(v)** a microbe–microbe interaction between the two symbiont genotypes that is detrimental to the host because the two bacterial genotypes can be incompatible with each other (i.e. cytoplasmic incompatibility); and **(vi)** a two-way nuclear interaction that leads to a breakdown in the immune system and the instantaneous acquisition of a new bacterial species (purple) that causes pathology, an outcome of the Hybrid Susceptibility Hypothesis. Alternatively, the two nuclear interactors can abnormally suppress beneficial bacteria and lead to hybrid problems owing to the lack of beneficial bacteria, such as an autoimmune response.

negative epistasis between immunity genes from different species and cause an inferior level of resistance than either parental species. This phenomenon is generally referred to as the hybrid susceptibility hypothesis, in which hybrids are more susceptible to infection by pathogens than are non-hybrids.

If pathogens have a detrimental effect on hybrid fitness, then they could be important in reducing hybrid fertility and viability. Three examples of F_1 viral activation in plant hybrids have recently been characterized, in which pathogenicity is rare or asymptomatic in parents but pathogenic in hybrids [50,51]. In addition, a comparative study of 162 plant and animal hybridizations found that even in the absence of controlling for environment or genetic divergence between parents, hybrid susceptibility was observed in 10–20% of the cases for insect herbivores on hybrid plants, 29% of the cases for fungal parasites on hybrid plants, and 50% of the cases in animal studies [52].

One of the most famous cases of hybrid susceptibility is from Dobzhansky's work on *D. paulistorum* [53]. Dobzhansky described several, morphologically indistinguishable, 'races or incipient species' that show varying degrees of sexual isolation and F_1 hybrid male sterility. He postulated, based on Lee Ehrman's and David Williamson's unpublished work at the time, that the sterility of these incipient species is 'due to its having acquired and become adapted to a new commensal or symbiont' [53]. Recently *Wolbachia* was identified as the

Box 3. Cytoplasmic incompatibility and speciation

Based on the Biological Species Concept, populations with identical genetic backgrounds are considered different species if they are isolated by bidirectional CI (Figure I). CI reduces or eliminates the production of F_1 hybrids and hinders gene flow between hybridizing populations. Thus, infection status can form the basis of a species diagnosis. Empirical evidence of bidirectional CI is taxonomically widespread, and occurs within species in *Culex pipiens* mosquitoes, various species of *Drosophila simulans* (reviewed in [11]), and between sympatric species of mites and species of *Nasonia* wasps [91,92]. Furthermore, theoretical evidence indicates that bidirectional CI can stably persist in populations that undergo high rates of migration [93,94].

The expression of CI can select for additional forms of RI through reinforcement (the process by which post-mating isolation acts as a direct selective pressure for the evolution of pre-mating isolation in areas of sympatry [12,14,95]). Pre-mating isolation is selected for because post-mating isolation is a wasteland for parental gametes: because hybrid offspring are dead or sterile, they cannot pass on genes themselves. *Wolbachia*-induced CI will have a strong effect on reinforcement. Consider a simple model in which hybrid fitness is reduced owing to CI in one scenario and a simple two-locus genetic incompatibility in the other. Because CI halts gene flow at the F_1 generation, whereas most genes involved in early genetic incompatibilities are recessive and limit gene flow in some F_2 genotypes or the heterogametic F_1 genotype (in accordance with Haldane's rule), *Wolbachia* has a higher likelihood of driving reinforcement [11]. The upshot is twofold. First, the F_1 isolation caused by CI reduces more gene flow by eliminating hybrids irrespective of their sex or genotype; second, F_1 isolation prevents recombination from slashing the required linkage disequilibria between the incompatibility locus and the mate-discrimination locus [11]. Recessive incompatibilities do not share this luxury because more fit hybrids will be produced and recombination in the previous generations can break down the required linkage disequilibria. Theoretical treatment supports this prediction [93] and empirical evidence from mushroom-feeding flies strongly supports reinforcement of pre-mating isolation by *Wolbachia*-induced CI [95].

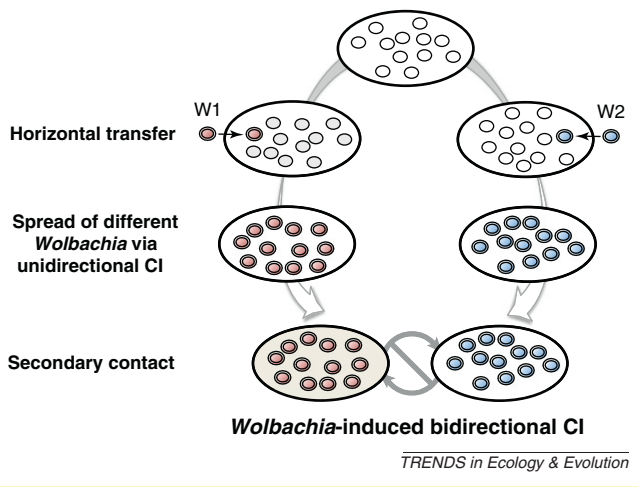

Figure I. Schematic of speciation by bacterial-induced cytoplasmic incompatibility. An ancestral population (large circle) of uninfected individuals (small open circles) splits into two populations that subsequently acquire different cytoplasmic, *Wolbachia* infections (colored circles labeled W1 and W2) by horizontal transfer. These single infections spread to fixation within each population by unidirectional cytoplasmic incompatibility (CI), a phenomenon that imparts a relative fitness advantage to infected females by causing embryonic death in crosses between infected males and uninfected females. Because infected females (the transmitting sex of cytoplasmic bacteria) are compatible with either infected or uninfected males, they do not suffer this fitness reduction; therefore, unidirectional CI can rapidly spread the bacteria through host populations. Upon secondary contact of these populations in sympatry or parapatry, bidirectional CI causes reciprocal incompatibility in both cross directions. Thus, species can arise without morphological or genetic divergence. Furthermore, F_1 bidirectional CI can select for the additional evolution of pre-mating isolation by reinforcement.

resident inhabitant of the testes and ovaries of the fly semi-species [18]. However, in hybrid males, these infections essentially become severe pathogens and cause F_1 male sterility. Furthermore, the original mate discrimination observed between the semi-species can be depleted by treating the *Wolbachia* antibiotically. A similar observation is true for *Heliothis virescens* and *Heliothis subflexa* moths, which harbor a bacterium that persists naturally in the guts of parental populations but overproliferates in hybrid testes and causes male sterility [54,55]. Taken together, these data specify that selective pressures related to host–pathogen conflict or symbiont maintenance can cause the breakdown of immunocompetence in hybrids and the evolution of gene flow barriers.

Hybrid autoimmunity

Negative epistasis between immunity genes in hybrids can also upregulate the immune system, suppress beneficial bacteria and cause hybrid maladies. Thus, a hybrid can turn its immune system on itself (i.e. autoimmunity), even in the absence of pathogens. The genes induced during hybrid autoimmunity correspond to the genes induced during the immune response to pathogen infection.

Although this subject has recently received attention, it is a common, incipient incompatibility described in the plant literature [12,56–59]. In a genetic dissection, plant defense genes in varieties of *Arabidopsis thaliana* were found to induce an autoimmune response in hybrid progeny, known as hybrid necrosis, as well as an abnormal suppression of inoculated pathogens [56]. These rapidly evolving genes encode nucleotide-binding domain and leucine-rich repeat (NB-LRR)-type immune receptors that function to recognize specifically a wide range of effector proteins from different pathogens and initiate downstream immune responses. Likewise, in a subspecies of *Oryza sativa* rice, the NB-LRR gene family causes an autoimmune-like phenotype [60]. Finally, *Rin4* is an immunity gene underlying HI in hybrids between the lettuce species *Lactuca sativa* and *Lactuca saligna* [61]. The incompatibility gene *Rin4* encodes a membrane-associated protein that is a negative regulator of basal defense and a target of effectors secreted by *Pseudomonas syringae* [62].

In summary, studies of hybrid autoimmunity reinforce the idea that features of the immune system can predispose defense genes to rapid evolution and, ultimately, the evolution of negative epistasis in hybrids. Although more research is needed, a bias of effector-triggered immunity genes in hybrid weakness would be persuasive evidence for symbiont-assisted speciation. For example, our own assessment of a 4450-gene study by Ranz *et al.* [63], demonstrated that approximately 93% of the immune genes in hybrid *Drosophila* species are irregularly expressed in comparison to 59% of the whole genome. HI between immune genes is essentially a speciation footprint of symbiosis.

Evaluating speciation by symbiosis

The challenge ahead for those studying symbiont-assisted speciation is to formulate a coherent theory of speciation

that includes both genes and symbionts, with evidence from empirical and theoretical investigations. We propose three ways in which these advances are likely to be made. First, comparative investigations will assess the relative role of symbionts in speciation using well-studied species pairs that permit a dissection of isolation barriers caused by symbionts and genes. Such studies could address the relative fraction of isolation owing to symbionts and whether taxa infected with certain microbial species show higher rates of speciation than do uninfected taxa. Second, empirical studies in the laboratory that test the strength of RI in conventionally reared and germ-free (i.e. antibiotically cured) hosts could determine the dependence of RI on the microbiota. For instance, an intriguing question would be: is hybrid lethality curable? Third, population genetic studies will need to assess whether symbionts accelerate the evolution of reproductive barriers and how their contribution compares to other causes of reproductive barriers. Our example in Box 2, Figure I highlights that symbionts can accelerate the evolution of HI, and this topic could be a fruitful area of research.

Concluding remarks

In this review, we organized and critically synthesized the literature on the microbiology of speciation to answer the following questions: why has symbiosis lurked in the background of most speciation research? Does the microbiota of a host directly induce RI and, if so, how frequently? Do microbial symbionts shape the evolution of nuclear-based RI, such as HI between immune genes? The data presented in this review equip microbiologists and evolutionary biologists with evidence of where symbiosis fits into the speciation field and vice versa. Moving forward, there are several areas that need further attention, including a cohesive, theoretical framework for the evolution of reproductive isolation by symbiosis, empirical studies of the relative role of symbionts versus genes in incipient speciation events across a wide range of fauna and flora, and the extent to which host species phylogenies parallel the relations of their microbiota. Arguably, the study of evolution is experiencing a significant fusion with microbial symbiosis. Multicellular organisms cannot exist in nature without their symbionts. The 20th-century pioneers of evolutionary biology would have been astonished to see what roles the microbiota play in eukaryotic evolution. We conclude with a suggestion; as biologists weave symbiosis into classic models of speciation, Wallin's 1927 synthesis [8] should be recognized for its rightful position as the initial and imaginative work on the microbiology of speciation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tree.2012.03.011.

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