

INTRODUCTION

Nature's microbiome: introduction

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In recent years, it has become common knowledge that we, and the many organisms around us, are symbiotic creatures, harbouring large numbers of internal and external microbial residents. Research on symbiosis has progressed remarkably since the days of van Leeuwenhoek and de Bary, whose discoveries paved the way for over two centuries of fascinating work. Indeed, since their findings on bacteria in human mouths and algal-fungal partnerships that constitute lichens, we have discovered that microbial symbionts have shaped the make-up of the eukaryotic cell and that they continue to influence growth, development, energy metabolism, nutrition, digestion and defence of eukaryotes from across the globe (Wernegreen 2012; McFall-Ngai *et al.* 2013). It is now widely understood that microbial symbionts are important sources of innovation across eukaryotes, making symbiosis one of the hallmarks of eukaryotic biology.

Historically, microscopy, analytical chemistry, antibiotic curing and a variety of other techniques were essential tools for the field, but recent innovations have propelled us into a golden age for symbiosis research. The identities and functions of both cultivable and noncultivable viruses, archaea, bacteria, protists and fungi can now be elucidated with genomic, transcriptomic or proteomic tools (Woyke *et al.* 2006; Warnecke *et al.* 2007; Engel *et al.* 2012; Kleiner *et al.* 2012; Sanders *et al.* 2013). Certain symbionts or hosts can be genetically modified, while some hosts can be manipulated via RNA interference, helping to understand the mechanisms behind symbiont colonization, persistence and functional contributions (Szeto *et al.* 1987; Dale *et al.* 2001; Radutoiu *et al.* 2003; Spiering *et al.* 2005). Furthermore, evolutionary histories of microbes and their hosts can be elucidated, revealing how commonly certain

microbes have converged on lifestyles as symbionts and how they have spread across hosts over time (Zchori-Fein *et al.* 2001; Russell *et al.* 2003, 2009; Canback *et al.* 2004; Degnan *et al.* 2004; Pochon *et al.* 2004; Moran *et al.* 2008; Sachs *et al.* 2009; Mondo *et al.* 2012). Importantly, these molecular tools permit the study of symbiosis beyond model systems, enabling us to learn a good deal about uncultivable symbionts from hosts that are not amenable to laboratory or greenhouse rearing or to experimental manipulation.

Perhaps no other field has been impacted by these new tools as much as human medicine, where it is now understood that nonpathogenic microbes play integral roles in health and immunity. The often-cited estimate of 100 times as many microbe- vs. human-encoded genes makes it clear that a good deal of novelty can be contributed by our microbiota (Backhed *et al.* 2005). But outside this field, nature's microbiome—the communities of microbes colonizing host eukaryotes (Lederberg & McCray 2001)—has long been viewed as an important source of evolutionary novelty (Douglas 1989; Margulis 1996; Cavalier-Smith 2002). And innovations made through cutting-edge molecular research in many non-human organisms can tell us much about ourselves as well as life in the world around us.

In this special issue of *Molecular Ecology*, we present 28 articles incorporating molecular and bioinformatics tools to dissect the intimate and prolonged associations that define symbioses. We have organized these studies into three sections, focused on (i) the composition of symbiotic communities and how this varies across hosts, tissues and development, and in response to environmental change ('The Dynamic Microbiome'); (ii) the roles that microbes play for their hosts and the underlying mechanisms behind these functions ('Microbiome Function'); and (iii) the nature and mechanisms of interactions between hosts and symbionts and between the co-inhabiting symbionts themselves ('The Interactive Microbiome'). These articles highlight the state-of-the-art in microbiome research, with novel discoveries for well-developed models and for other budding systems beyond the human realm.

The dynamic microbiome

Correlates of community variation

Most symbiotic microbes do not exist in isolation, instead co-inhabiting their eukaryotic hosts alongside other microbes. Central to the study of these symbiotic communities is the need to catalogue the variation in presence, abundance and taxonomic composition across different hosts (beta diversity). Knowing how communities vary yields insights into the stability of symbioses. Furthermore,

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through well-replicated study design (Prosser 2010; Knight *et al.* 2012) and deep sampling of communities, correlations between community composition and host phylogenetic relatedness, geography and ecological traits can help to illuminate the causes and consequences of host–microbe associations.

Such an approach is taken by the first study reviewed for this issue. This article focuses on bacterial communities of importance for the world's amphibians, which have been severely impacted by the pathogen *Batrachochytrium dendrobatidis*. Prior work has shown that skin-dwelling microbes can help to defend against 'chytrid' fungus, through the production of antimicrobial compounds (Woodhams *et al.* 2007; Harris *et al.* 2009). And preliminary studies suggest the potential for their use in natural populations to effectively immunize them against the pathogen (Bletz *et al.* 2013). Here, Kueneman *et al.* (this issue) take an important step in characterizing the microflora of amphibian skin. Through the use of 16S rRNA amplicon sequencing (with Illumina technology), they find substantial differences in skin bacteria from three different amphibian species when controlling for geography, building on results from a prior study (McKenzie *et al.* 2012). They also note shifts in community composition across development and subtle differences between populations from different regions. Differences across host species and developmental stages suggest important considerations for future studies on bacterial inoculation strategies to defend amphibians against chytrid; they also provide some of the deepest insights yet into the cutaneous microbiomes of amphibians.

Kaltenpoth and Steiger (this issue) perform a similar study of microbial community variation across burying beetles from two subfamilies within the Silphidae. Little was known about the gut microbiota from these flesh-decomposing beetles before this study, and thus, there had been few insights into the types or functions of gut microbes. But interestingly, through amplicon sequencing of 16S rRNA, the authors find a good deal of similarity in the types of gut bacteria from different beetle species, especially those from the same subfamily. Also widespread were ascomycetous yeast symbionts related to those from the genus *Yarrowia*. Like some of the identified bacterial symbionts, these too fell into host-specific lineages. So when combined with the estimated age of the hosts' common ancestor, these patterns hint at ancient (>100 million year) and specialized interactions between burying beetles and microbes of likely functional importance.

Similar in nature to the conserved community composition among burying beetles are discoveries from a diverse and modestly (>65 mya; see Price *et al.* 2014) group of Neotropical ants. In their study of adult workers from the tribe Cephalotini (including numerous specimens from the *Cephalotes* genus), Sanders *et al.* (this issue) illustrate that these ants have engaged in stable associations with a core group of bacteria from several ant-specific lineages. The stability of these interactions across tens of millions of years is somewhat similar to Kaltenpoth and Steiger's (this

issue) finding from burying beetles, with both differing notably from the more ephemeral nature of gut symbionts from several invertebrates (Engel & Moran 2013; Wong *et al.* 2013). While anal secretions into the larval food source are argued to promote vertical transfer and partner fidelity in the beetle system, oral-anal trophallaxis is thought to passage *Cephalotes* gut symbionts among siblings and generations. The resulting partner fidelity should favour mutualism and, if stable over long periods, could also lead to codiversification between hosts and microbes. Kaltenpoth and Steiger (this issue) provide suggestive evidence for this (i.e. trends of congruence between gut community similarity dendrograms and the host phylogeny), so do Sanders *et al.* (this issue), who develop a novel statistical tool towards this end that should be of use to the broader community (see Box 1 for more detail). Interestingly, Sanders *et al.* (this issue) findings suggest a greater signal of codiversification for *Cephalotes* gut microbes than that seen in their re-analysis of a great ape gut microbiome data set (Ochman *et al.* 2010), suggesting differences in the stability of gut microbiota due to differing mechanisms of acquisition.

Hu *et al.* (this issue) contribute an additional study on the *Cephalotes* symbiont system, focusing on intraspecific variation in gut communities across *C. varians*. In this investigation, communities from adult workers are shown to vary subtly across colonies, with shifts in the relative abundance of 'core' bacterial species, defined here as 97% OTUs present in over two-thirds of sampled ant colonies. However, assessment of 16S rRNA genotypes from their amplicon sequence pools reveal that bacterial strains differ in abundance or presence/absence across colonies (see Box 1 for more detail). Factors such as diet, host genetics, genetic drift, inoculum composition and host-level selection are posited to play roles in such variation, and the impacts of diet were tested in this work. But of the seven administered diets, only one—pollen—led to repeatable alterations in gut microbiota across colonies. As no bacteria were detected in pollen, the proliferation of a specific Rhizobiales strain in worker ants consuming pollen diets was argued to be a shift in the indigenous microbiota. This suggests one driver of natural community variation, albeit one that is not likely to explain most between-colony differences. The finding also hints at the potential use of compounds in pollen to the benefit of Rhizobiales and at their potential role in pollen digestion. Given the importance of this substance in the diets of many *Cephalotes* ants, and the broad distribution of these bacteria across this group, it will be intriguing to assess whether their gut symbionts have played a substantial role in the evolution of this feeding habit.

Diet features prominently in the study by Delsuc *et al.* (this issue), who use amplicon 16S rRNA sequencing to compare the distal gut microbiota from ant- and termite-feeding mammals, dubbed 'myrmecophages'. After removing problematic libraries suspected of contamination (see Box 1 for more on their source-tracking method), they compare faecal communities between

Box 1: Major challenges and discoveries addressed in this issue

Within the last decade, the costs and yields of molecular technologies have improved drastically, as have the variety and accessibility of bioinformatic tools required for the analysis of microbiome data. Thus molecular-based symbiosis research, including that on microbial communities and symbiont genomes, can now easily meet the standards and statistical rigour required for sound science (Prosser 2010). Below we highlight some of the challenges for current symbiosis research that have been addressed by studies in this issue through their analyses of next-generation sequence data sets, including both amplicon and full-scale 'omics approaches.

- Challenge: How do symbionts evolve over short timescales?
- Approach: Brown *et al.* (this issue) sequence genomes from *Ishikawaella* symbionts in stinkbug hosts collected at different times and in different locations. Their population genomics approach gives insights into symbiont evolution immediately after their hosts' invasion and establishment as soybean pests in the United States.
- Discovery: The authors find that allele frequency shifts for *Ishikawaella* have been most drastic for several genes from nutritional pathways, including riboflavin biosynthesis. It appears likely, then, that positive selection has acted on symbiont nutritional function since the time of the stinkbug's arrival in 2009.

- Implications: While it is widely recognized that symbionts shape host evolution, there is little understanding of how they do so over short timescales in nature. Population genomics is suited to address this question and will become more accessible with continuing declines in the costs of DNA sequencing.

- Challenge: How is symbiont function regulated to meet host need?
- Approach: Real-time quantitative PCR targeting toxin genes encoded by defensive symbionts (Hamilton *et al.* this issue; Martinez *et al.* this issue).
- Discovery: The two focal studies found no clear or intuitive transcriptional up-regulation of defensive toxin genes in response to natural enemy attack. In Martinez *et al.*'s (this issue) study, phage-mediated symbiont lysis appeared to be triggered by parasitoid wasp attack. This was put forth as the key step of functional regulation as it likely enables toxin delivery into the hemolymph, where developing wasp larvae are found.
- Implications: While gene expression for some symbionts may respond to host-level challenges (Loroch *et al.* 1995), the regulation of symbiont transcriptomes may not be tight or intuitive in some systems (Wilcox *et al.* 2003). In these cases, the functional regulation of symbionts may involve alternative mechanisms (Burke *et al.* 2010). For instance, phloem-feeding insect hosts may regulate symbiont nutrient provisioning by supplying the missing ingredients in biosynthetic pathways otherwise encoded by their microbes, as discussed by Hansen & Moran (this issue).

- Challenge: How many microbial strains and species colonize eukaryotic hosts? Alpha diversity appears inflated, due in part to PCR and sequencing error. Conversely, analyses of most next-generation sequencing data sets have ignored diversity involving closely related microbes.
- Approach 1: Jing *et al.* (this issue) use a Poisson-based model to calculate the probabilities that 'minor' OTUs (97% similarity at 16S rRNA) arise due to chance, given predicted rates of sequence error.
- Approach 2: After making manual alignment adjustments, Hu *et al.* (this issue) compute genotypes for all 16S rRNA sequence reads from their major OTUs. Genotypes consisted of nucleotides at variable sites, that is, those where the minor allele had at least 1% frequency for the given pool of sequences belonging to the single OTU.
- Discovery 1: The authors find that the numbers of 97% OTUs likely from 'real' organisms is ~2- to 5-fold lower than the number calculated from an uncorrected data set.

- Discovery 2: Gut communities consisted of ~10–20 97% OTUs, but most were made up by multiple strains, suggesting an excess of 50 types of bacteria per worker ant gut. Strains varied significantly and substantially across host ant colonies, and across regions and diets, suggesting the potential for important cryptic variation.
- Implications: While 'de-noising' (Quince *et al.* 2009) helps to minimize problems of OTU inflation, other approaches must be applied to improve the accuracy of alpha diversity estimates from amplicon or shotgun sequencing data. This will allow us to better study just how and why bacterial diversity varies across different hosts. Furthermore, hidden strain diversity can be detected with newly automated analysis (Eren *et al.* 2013) or sequencing tools (Faith *et al.* 2013). The study of strain distributions should be routinely instituted in analyses of next-generation sequencing data sets and will be important for understanding the ecology and evolution of host–microbe symbioses (Faith *et al.* 2013). Importantly, studies that move beyond a 16S rRNA focus (Schloissnig *et al.* 2013) will be most successful in identifying variation between strains due to the slowly evolving nature of this gene.

Box 1 (continued)

- Challenge: How faithful are symbioses over macro-evolutionary timescales? Do microbes codiversify with their hosts? These are not easy questions to address for multispecies microbial communities.
 - Approach: Sanders *et al.* (this issue) calculate gut community similarity across *Cephalotes* ant species based on the sharing of 99% and, separately, 97% OTUs. Their estimates of community similarity are made using Jaccard index estimates based on 16S rRNA pyrosequencing data.
 - Discovery: Community similarity dendrograms calculated based on 99% OTU distributions showed significant congruence with the host ant phylogeny. Yet congruence with the host phylogeny was minimal for dendrograms estimated from 97% OTU sharing. This supports a history of host–symbiont codiversification (vs. phylogenetically correlated environmental filtering), as small levels of 16S rRNA divergence (<1.5%) are expected for codiversifying microbes given the age of this host group.
 - Implications: Calculation of individual symbiont phylogenies (vs. community similarity dendrograms) is still the gold standard for codiversification studies. But it is an elusive goal for diverse symbiont communities. Thus, Sanders *et al.*'s (this issue) new clustering width sensitivity method provides one of the best current approaches. The broad range of sampled host species and use of a rigorously generated host phylogeny in this study also serve as a model for future research on the histories and fidelity of host–microbe symbioses.
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- Challenge: How can one detect sequence libraries plagued by contamination?
 - Approach: In their study on ant- and termite-eating mammalian gut communities, Delsuc *et al.* (this issue) use a source-tracking method to identify libraries with likely contamination. Using Bayesian statistics, they estimate the percentage of reads in each sequence library that can be assigned to soil or mammalian gut habitats, using sequences from several previously studied soil and faecal samples as representative source data sets. Sequence libraries with over 10% assignment to soil communities or with less than 0.01% assignment to mammalian gut communities were removed from the analysis.
 - Discovery: Of 92 generated sequence libraries, 27 were removed. Five of those removed had over 10% of their communities assigned to soil habitats. The rest had less than 0.01% assigned to mammalian guts. A good number of samples removed were suspected of possible contamination in advance of sequencing due to soil contamination or exposure in the field for possibly substantial durations before collection.
 - Implications: The application of this method seems especially important for studies in which there has been no sampling of sequence libraries from 'negative' controls (an omission that will ideally become less common) and for which newly described communities are being identified (especially those sampled with few biological replicates). The range of contaminating microbes will differ depending on the host habitat, revealing a need for future generation of source libraries for marine and freshwater habitats, and beyond. It will also be useful to develop source communities for common laboratory- and extraction kit-dwelling microbes, to prevent publication on 'symbiotic' communities confounded by methodological (vs. field) contamination.

myrmecophages and to those from other mammals to determine whether this diet is associated with a particular type of gut microbiota. Aardvarks, anteaters and aardwolves—three independently derived myrmecophages—are found to share fairly similar gut bacteria, in spite of substantial evolutionary divergence between them. Moreover, each harboured gut bacterial communities that were distinct from those of their relatives. While similar microbial convergence has been seen for mammals at the same trophic level (Ley *et al.* 2008; Muegge *et al.* 2011), this study provides the first example for microbiome convergence among mammals with such a specific, shared diet. The authors discuss the possible implications of these findings, including the possibility of beneficial contributions such as chitin digestion.

In addition to diet and phylogeny as correlates of symbiont community composition, both the Hu *et al.* (this issue) and Kueneman *et al.* (this issue) articles report on variation in communities from the same host species across sites.

Coats *et al.* (this issue) detect a similar trend for the microbes from the rhizosphere soil of invasive Japanese barberry plants. Their analyses of bacterial 16S rRNA and fungal ITS sequences suggest differences in rhizosphere composition across three sites in coastal Maine. These sites differ in the types of surrounding dominant plants, suggesting a potential cause of rhizosphere differences that may be important for barberry fitness.

Filtering of environmentally acquired symbionts

Many microbial symbionts can persist outside of their hosts, which obtain them from the environment. But it is very often the case that the microbes proliferating in the symbiotic context are distinct from those that dominate the surrounding free-living environment (e.g. Hu *et al.* this issue; Kueneman *et al.* this issue). When symbionts are acquired environmentally, this suggests the action of environmental filtering mechanisms, which are largely

undescribed in most systems. In this issue, Garcia *et al.* (this issue) report such a study of environmentally acquired *Burkholderia* bacteria from the guts of broad-headed bugs. Experimental evidence from their publication suggests beneficial effects conferred by these bacteria, which are common across this group of hemipterans. Sequencing of bacterial 16S rRNA from gut bacteria across four host bug species from 12 locations in the southeastern USA showed that *Burkholderia* dominate the gut and that distinct host species harbour similar *Burkholderia* from the same 97% OTUs. The lack of strong specificity at the 16S rRNA level suggests that partner fidelity may not be strong for these symbioses. But environmental filtering appears likely, as a limited range of *Burkholderia* from mostly bug-specific clades dominate the guts of these insect hosts. The authors also find that the symbiotic *Burkholderia* can survive when exposed to hemolymph from immune-challenged bug hosts in culture-based assays, although this was also true for nonsymbiotic bacteria. Thus, while not the sole explanation, symbiont resistance to host immune defences could play a partial mechanistic role in the suspected environmental filtering.

Similar filtering is seen in a study by Moitinho-Silva *et al.* (this issue), who examine bacteria from sponge species and the surrounding seawater. In this investigation, the authors focus on two types of sponge hosts, which differ drastically in microbial biomass: high microbial abundance (HMA) sponge species not only contain many more microbes (with up to 35% of their biomass made up by microbes), but also harbour more diverse communities of bacteria and archaea compared with their low microbial abundance (LMA) sponge counterparts. Moitinho-Silva *et al.* (this issue) use 454 amplicon pyrosequencing of 16S rRNA genes and transcripts to compare the diversity, specificity and transcriptional activity of microbes associated with one LMA sponge species, one HMA species, and surrounding seawater from the same sampling site in the Red Sea. While their study reaffirms the higher bacterial diversity of HMA host sponges, the LMA sponge is shown to harbour a specific microbiota, contradicting the assumption that the bacterial community of LMA sponges resembles that of the surrounding environment. Also noteworthy is that this study is the first to examine the activity of Poribacteria, bacterial symbionts previously thought to only occur in sponges. The authors find Poribacteria in very low abundances in the surrounding seawater, where they appear to be metabolically inactive. Of general importance for hosts that harbour microbes that also occur in the environment are the authors' thoughts on a valid term for such symbionts: they argue that these should be called host-enriched rather than host-specific microbes, a concept that could be extended to other microbiota such as those that live in animal guts or the plant rhizosphere.

Symbiont response to environmental perturbation

The importance of symbionts to host fitness suggests that understanding symbiont responses to environmental

perturbations and gradients may be critical to predicting the host response. Studies at the forefront of symbiosis research are grappling with the coupled dynamics of host and symbiont responses to external perturbations, an activity of great importance during current times of human-driven environmental impact. For example, Dean *et al.* (this issue) investigate the effects of N-deposition on root-associated fungal symbionts in an alpine tundra ecosystem. Their work is among the first to show responses in fungal symbiont assemblages that parallel plant species responses to perturbation. Interestingly, the fungal assemblages of the plant species that was unresponsive to nitrogen addition showed the largest changes with fertilization, suggesting that shifts in microbial composition may buffer hosts from the effects of external perturbations (see also Lau & Lennon 2012). For the plant species that strongly declined in response to nitrogen addition, nitrogen also caused a large reduction in the dominant clade of root-associated fungi (Helotiales) and an overall increase in fungal diversity. This result may indicate the loss of an important fungal mutualism under high-nitrogen conditions. Finally, plant-removal experiments showed that the presence of a neighbouring host species can alter how the microbial community responds to external perturbation, highlighting the potential for interactive microbiome effects.

Gehring *et al.* (this issue) focus on drought as an external perturbation and similarly use plant-removal experiments to complement understanding of fungal responses to global change. Here, the authors' key contribution is in comparing the relative importance of host genetic variation, abiotic and biotic stressors on host-associated microbial communities. Notably, the authors found strong evidence for interactive host genotype X environment effects on microbial composition. Ectomycorrhizal (EM) fungal assemblages on host piñon pine genotypes that were susceptible to herbivory remained largely stable under increasingly dry conditions, but fungi associated with plant genotypes resistant to herbivory shifted strongly. In contrast, the removal of neighbouring shrubs altered fungal communities of insect-resistant trees, but not insect-susceptible trees.

Oliveira *et al.* (this issue) also investigate the effects of external perturbations on host-associated microbes. Here, the degree of petroleum hydrocarbon contamination altered endophytic bacterial assemblages found in the roots of salt marsh plants, with characteristic plant-species-specific 'imprints' in bacterial community structure. Importantly, PAH dioxygenase genes involved in the breakdown of hydrocarbon pollutants were discovered in the endophytic bacteria of roots of two common plant species. Some of these occurred on plasmids, which could allow for horizontal gene transfer, although this was only documented from the most contaminated habitats assessed in this study. These findings indicate that host-associated microbes, such as endophytic bacteria, have high potential to degrade environmental toxins, such as petroleum hydrocarbons, perhaps ultimately benefiting their hosts. Approaches used here, such as hydrocarbon-enriched substrates for culturing and 'baiting' functionally significant

taxa, may help to reveal similar functions for other plant-associated microbes.

Distributions and relatedness of symbionts across related invertebrates

Symbioses between sulphur-oxidizing bacteria and animals are well known from marine environments (Dubilier *et al.* 2008). In freshwater, the only well-described symbiosis of this type is between ectosymbiotic sulphur-oxidizing bacteria of the genus *Thiothrix* and amphipod niphargid hosts from sulphide-rich caves in Italy (Frasassi caves). Initial work on the Frasassi symbioses suggested that these associations evolved several times, an hypothesis examined here by Flot *et al.* (this issue) who widened the sampling area to various subterranean environments in eastern Romania. The authors detected *Thiothrix* bacteria on all seven examined host species. Interestingly, there were a number of similarities in the morphology, phylogeny, and distribution patterns of the Romanian and Italian symbionts despite the fact that the hosts from these two locations are not closely related. These parallels in hosts separated by more than 1200 km suggest that these associations may be much more widespread than currently recognized.

In another study, Altamia *et al.* (this issue) estimate evolutionary divergence among *Teredinibacter turnerae* symbionts from wood-feeding bivalve species in the family Teredinidae (shipworms). These bacteria occur intracellularly in the gills of the shipworms where they produce enzymes, including cellulases and nitrogenase, which may contribute to the host's ability to survive on an indigestible and nitrogen deficient diet of wood. Previous phylogenetic analyses of 16S rRNA sequences from a limited number of *T. turnerae* isolates (6) across host species detected no sequence variation (Distel *et al.* 1991), raising the question of whether *T. turnerae* represents a single symbiont population with worldwide distribution. The authors address this here through the analysis of sequence diversity across six bacterial genes among 25 isolates of *T. turnerae*. In total, symbionts from 13 shipworm species hailing from 15 locations in the Atlantic, Pacific and Indian Oceans clustered into two well-supported clades showing some host specificity: each host shipworm species harboured symbionts from just one of these two lineages. The observed specificity was independent of host geography and time of sampling, indicating that the associations between individual host species and their respective *T. turnerae* clades are stable over long periods and great distances of geographical separation.

Understanding diversity within communities

Alpha diversity seems to vary across the microbiomes of various plants and animals, which themselves tend to harbour fewer numbers of species and higher order taxa compared with habitats such as soil or water. Insects harbouring bacteriocyte-dwelling primary symbionts, in contrast, appear depauperate in their numbers of asso-

ciated microbial species. Accordingly, in this issue, Jing *et al.* (this issue) perform a rigorous assessment of alpha diversity in the gut communities of whiteflies and their phloem-feeding relatives through high-depth sequencing of 16S rRNA amplicons. Impressively, they find only 3–7 97% OTUs in most of the examined sap-feeding insects, a trend consistent across insects from the field and the laboratory. While this diversity could directly extend from the rarity of bacteria in the insects' diets of phloem sap, it is also possible that intrinsic biological attributes of these sap-feeders are of importance. Indeed, it will be interesting to determine whether insects that have independently evolved highly integrated symbioses, like those seen in phloem-feeders (e.g. grain-weevils or various blood-feeding insects), are similar in harbouring low diversity gut communities or whether this is a more general phenomenon across other insects (e.g. in *Drosophila* as shown in Jing *et al.*'s manuscript).

Dynamics across tissues and development

While other studies in this section address symbiotic diversity within individuals, and across host lineages and populations, Kikuchi and Fukatsu (this issue) describe variation in symbiont establishment and population densities across host development. In their system, environmentally acquired *Burkholderia* symbionts, with known impacts on fitness and insecticide resistance, are found in particular regions of the bean bug (*Riptortus pedestris*) gut. But as for many other gut symbioses, the mode and tempo of colonization during development are not well understood. Using a genetically engineered *Burkholderia* symbiont expressing green fluorescent protein (GFP), the authors perform fluorescent microscopy and qPCR to assess the timing of colonization and symbiont proliferation. Their findings suggest that establishment occurs quickly, with bacteria arriving at the symbiont-housing portion of the midgut within six hours of inoculation. Population sizes then expand by over four orders of magnitude before plateauing 3–4 days post-inoculation. Colonization dynamics are fairly similar under a range of conditions, including acquisition from soil and cultivation media spiked with low symbiont titres, although lower density inocula appear to delay the rapid population build-up within the midgut. While these contributions are fairly novel, perhaps even more exciting is the promise this system holds as a model for animal-gut microbe symbioses. Indeed, very few symbionts can be cultivated and genetically manipulated. Thus, the capacity to inoculate bean bug hosts with transgenic bacteria should yield many opportunities for studies on the mechanisms underlying symbiosis.

While Goffredi *et al.* (this issue) similarly place an emphasis on microbial shifts across development, they also consider differentiation of symbiotic communities across host tissues of the deep-sea crab *Kiwa puravida*. These fascinating decapod crustaceans were first discovered at hydrothermal vents only nine years ago and were dubbed yeti crabs because their legs are covered with specialized setae

that are densely colonized with filamentous ectosymbiotic bacteria. How these ectosymbionts are transmitted from one generation to the next and the reasons why they are found in particular locations on the body surfaces of their hosts are not well understood. Through their use of morphological and molecular methods, the authors show that (i) the microbial community associated with the yeti crab is more complex than previously assumed; (ii) the pereopods (legs) of the crab are associated with the least diverse microbial assemblage compared to the rest of the body; and (iii) the core microbial community is present on eggs, juveniles and adults, although community composition and diversity vary between these three life stages. The similarity in some of these ontogenetic patterns with those from the association between hydrothermal vent shrimp and their ectosymbiotic communities (Guri *et al.* 2012) suggests that there may be a certain degree of convergence in symbiosis development in deep-sea crustacean hosts.

Microbiome function

Throughout their billions of years on this planet, bacteria have invented a wide range of metabolisms that are not encoded by the genomes of most eukaryotes, including both carbon and nitrogen fixation, and the utilization oxygen for aerobic respiration. But since their origins, eukaryotes have come to benefit from these processes through the acquisition of microbial symbionts. These functions are certainly some of the most profound among the services performed by microbes. But attributes such as nitrogen and phosphorous uptake, defence, nutrition and digestion have also played large roles in eukaryotic ecology and evolution, often opening new niches for eukaryotic organisms. Symbiosis can, thus, be seen as a major source of evolutionary novelty across the eukaryotes, perhaps taking the place of lateral gene transfer seen commonly in bacteria (e.g. see Oliver *et al.* 2010). In the below section, we discuss the results of several studies focused on the benefits of mutualistic symbionts along with their underlying mechanisms, providing a glimpse into the spectacular diversity of symbiotic innovations from across the world's eukaryotes.

Host plant utilization by herbivorous animals

A wide variety of animals consume plants as part of their diets. But plant-based resources may be hard to digest (wood and foliage), poor in essential amino acids (phloem and xylem sap) or well defended via secondary metabolites. Bacteria, protists and fungi are known to help some animals overcome these challenges, but it is not clear whether these symbioses drive the specificity of animal-plant interactions (Janson *et al.* 2008). Hansen and Moran (this issue) consider this issue in their review on the importance of bacteria to host plant utilization and specificity across insects. They first consider bacteria confined to specialized 'bacteriocyte' cells of sap-feeding hemipterans, which provide hosts with essential nutrients that are rare in their diets. These symbionts are required by their hosts

and are often ancient and highly specific associates. Due to their ubiquitous associations with the xylem- and phloem-feeding lifestyles, such microbes are now universally accepted to have played major roles in the colonization of these dietary niches (Moran & Baumann 1994). However, Hansen and Moran (this issue) point out that the genomes of these microbes show a good deal of conservation, with little capacity for genomic novelty via lateral gene transfer and little variation in the presence of nutrient biosynthetic pathways among relatives. On top of this, it is argued that their bacteriocyte localization would at least be highly inconvenient if these bacteria were to play roles in detoxification or responses to plant defence. It is, thus, argued that these symbionts are not likely to mediate specific interactions between insects and plants that underlie host plant range and specificity. Instead, the authors posit that microbes from the plant surface or insect gut associated may be more important in driving specific interactions between insect herbivores and their food.

Indeed, Humphrey *et al.* (this issue) suggest just such a role for plant-associated microbes, finding that leaf-surface bacteria can affect rates of insect herbivory indirectly, by eliciting plant defensive pathways. Specifically, experimental inoculations of phyllosphere *Pseudomonas* spp. increased the susceptibility of a native bittercress plant to leaf-mining flies from the genus *Scaptomyza*. In addition, bacterial abundances on leaves were naturally higher in herbivore-damaged than in undamaged leaves. There was strong bacterial specificity in the outcomes of these interactions. Some bacterial strains were negatively (rather than positively) associated with herbivory, and flies showed strain-specific preferences in bacterial inoculation choice tests on leaves. This study is particularly valuable for using a natural system to show the relevance of interactions between phyllosphere bacteria and herbivores in a field setting, because prior work has focused on model systems under controlled conditions.

In accordance with another prediction by Hansen and Moran (this issue), microbe-driven host plant utilization has recently been described for midgut-colonizing *Ishikawaella* symbionts from *Megacopta punctatissima* stinkbugs. Fascinatingly, experimental transfer of *Ishikawaella* symbionts from *M. punctatissima* enables soybean utilization by the congener *M. cribraria* through unknown mechanisms (Hosokawa *et al.* 2007). Brown *et al.* (this issue) adopt a population genomics approach to test whether *Ishikawaella* symbionts from invasive *M. cribraria* have enabled it to become a soybean pest since its arrival in the United States. By sequencing symbiont genomes from stinkbugs collected at different times and distances from the invasion site, the authors find few differences between *Ishikawaella* of invasive *M. cribraria* and the pest-conferring *Ishikawaella* of *M. punctatissima*. *Ishikawaella* genomes have evolved little since the invasion, with no fixed differences over time or between insects from different host plants. These findings suggest that these insects arrived in the USA as pests, with their capacities to use soybeans likely stemming from their specific gut symbionts.

Digestion and nutrition

Outside of these stinkbugs, gut bacteria are known to play important roles in the use of dead and decaying plant tissue by termites. While not an issue of host plant use, per se, these contributions are certainly essential to the ecosystem services contributed by detritivorous termites across the world's terrestrial habitats. The capacity to cultivate termite gut microbes has been important in elucidating the functions of particular symbionts. And, indeed, spirochaete symbionts present one such example, with *in vitro* discoveries previously showing their contributions to acetogenesis (and, thus, energy metabolism) and N₂ fixation (Leadbetter *et al.* 1999; Lilburn *et al.* 2001). Here, Lucey and Leadbetter (this issue) further study the metabolism of two strains of the cultured spirochaete *Treponema primita*, to investigate their potential contribution to lignin digestion. The authors show that lateral gene transfer has contributed genes potentially enabling the metabolism of aromatic compounds, such as those derived from the initial breakdown of lignin. Accordingly, aromatic ring cleavage is demonstrated here through a series of biochemical experiments. This process requires oxygen, revealing a means by which these anaerobes can tolerate and even use oxygen to drive the further breakdown of lignin by-products. Thus, while providing some of the first evidence for lignin degradation by gut bacteria of lower termites, the authors also elucidate novel microbial metabolisms of potential importance for carbon turnover.

Like termites, which receive nutrients from nitrogen-fixing symbionts, many creatures from the deep sea obtain nutritional provisions from symbionts. Hydrothermal vent tubeworms provide one example. These invertebrates lack a mouth and gut and depend on their chemosynthetic bacterial symbionts for carbon, nitrogen and other nutrients. Tubeworms are found in a broad range of habitats, and variations in their body phenotype (long and skinny versus short and fat) have been hypothesized to be related to differences in their environment. Given the wide range of nitrogen concentrations in tubeworm habitats, Liao *et al.* (this issue) ask how tubeworm phenotype and environmental conditions influence nitrogen metabolism in these symbioses. The authors observe site-specific differences in the expression of genes used by the symbionts for the anaerobic respiration of nitrate, but host phenotype also played a role, for example in the pathways used for the assimilation of nitrogen from the environment. This study nicely highlights the physiological variability of highly related symbionts within a host population and shows how challenging it is to understand how host and environmental variability affect microbiota.

Defense

While nutritional and digestive roles of symbionts have been recognized for some time, it has more recently become appreciated just how widespread defensive services are across nature's microbiome (Oliver *et al.* 2014). Important groundwork for this research was laid out in marine invertebrate and terrestrial plant systems, and it is

now known that microbial defenders can have impacts at the ecosystem level (Clay *et al.* 2005). Insects have recently become a target for defensive symbiosis research (e.g. Hoffmann *et al.* 2011; Łukasik *et al.* 2013), due to findings of bacterially mediated protection in blister beetles, aphids, psyllids, fruit flies and mosquitoes; and two papers in this issue focus on bacterial defenders of these invertebrates. The first of these, by Hamilton *et al.* (this issue), aimed to discover the mechanisms of symbiont-driven defence through RNAseq studies of transcriptomes. By measuring gene expression in fruit flies (*Drosophila neotestacea*) with and without defensive *Spiroplasma* symbionts, and in the presence or absence of parasitic nematodes, the authors discover few effects of *Spiroplasma* on host-encoded immune gene expression. While, thus, ruling out host immune priming as the means of defence (Moreira *et al.* 2009), the authors discover the expression of two *Spiroplasma*-encoded toxin genes. As proteinaceous toxins have been implicated as defensive agents in other protective symbioses, these findings reveal a plausible mechanism behind prior discoveries of reduced nematode size and restored fly fertility in *Spiroplasma*-bearing hosts (Jaenike *et al.* 2010).

The interactive microbiome

Symbiosis is inherently an interactive process. Bacteria, protists and fungi that colonize plant or animal hosts often exist in multispecies and even multikingdom microbial ecosystems. These have their own ecologies that are influenced by host attributes, such as morphology and physiology. Within these ecosystems, microbes' interactions with hosts, with each other, and with their internal host environment will often shape their own evolution along with that of their hosts. In the below section, we highlight studies on the nature of these interactions along with those investigating the underlying mechanisms responsible for symbiont persistence and partner choice in symbiotic mutualisms. We also review one study on potential causes or consequences of symbiosis observed within host genomes.

Microbe–microbe interactions

In this section, Bakker *et al.* (this issue) first reconceptualize the dominant paradigm for host–microbe interactions, by moving away from a purely pairwise (host–microbe) perspective towards inclusion of both smaller-scale microbe–microbe interactions and large-scale host–host interactions. They use communities of *Streptomyces* in prairie soils varying in host plant species richness to show how host plant species identity and host species richness at the community scale can alter networks of coassociation among bacterial taxa. Notably, they link microbial composition to function and show that natural patterns of coassociation correlate with the antagonistic activities of *Streptomyces* in culture. By additionally examining the relative importance of soil edaphic factors in microbial composition, Bakker *et al.* (this issue) develop a new framework for understanding host microbiomes across multiple scales.

Engelmoer *et al.* (this issue) further develop the theme of microbe–microbe interactions using artificial host root systems to investigate competitive interactions between two closely related species of arbuscular mycorrhizal fungi. Competitive interactions between the fungi reduced overall fungal abundance, suggestive of potential costs to antagonistic interactions that occur within the microbiomes of hosts. Competition for resources was more intense inside of roots than in the extraradical matrix. The presence of competitor fungi also shifted how individual fungal species invested in intraradical vs. extraradical hyphae.

Martinez *et al.* (this issue) have also dissected the ecologies of host-associated microbes, and their responses to the environment, focusing their efforts on the responses of symbiont communities to natural enemy attack. In their system, heritable *Hamiltonella defensa* bacteria bearing ‘APSE’ bacteriophage protect pea aphids (*Acyrtosiphon pisum*) against parasitoid wasps (*Aphidius ervi*). Through a series of qPCR experiments, the authors quantify the densities of *H. defensa*, APSE and the nutritional symbiont *Buchnera* across aphid lines and in the presence and absence of parasitoid wasps. While the effects of parasitism were not consistent for all symbionts across all aphid lines, APSE bacteriophage titres consistently increased in response to parasitism, while APSE/*H. defensa* ratios rose as well. It is posited that this reflects phage-mediated bacterial cell lysis, previously hypothesized to deliver toxins to the hemocoel and the developing wasp (Moran *et al.* 2005). As parasitism did not consistently up-regulate phage-encoded toxin genes, lysis and release of existing toxins may instead serve as the major mechanisms behind protection.

Potential effects on host genome evolution

A very different approach to studying the interactive nature of symbiosis was taken by Duncan *et al.* (this issue), who investigated genomic attributes of sap-feeding hemipteran insects harbouring highly specialized nutritional symbionts. While symbiosis can drastically shape genome evolution of the bacterial partners (Moran & Wernegreen 2000; Hansen & Moran this issue), Duncan *et al.* (this issue) find that host genomes may also respond to these long-standing symbioses in important ways (see Dunning Hotopp *et al.* 2007 and Husnik *et al.* 2013 for different examples). Building upon a prior study that focused on aphids (Price *et al.* 2011), the authors expand a search for amino acid transporter gene across a range of other sap-feeding insects. Through a combination of genomic data mining, transcriptome annotation and phylogenetics, they identified and studied several homologs of transporter genes from gene families expanded in the pea aphid genome. Overall, the number of transporter genes was higher in phloem-feeding hemipteran insects than those with other diets from outside this order. The one examined xylem feeder (i.e. a cicada), found in a different suborder (Auchenorrhyncha) from the phloem-feeders (Sternorrhyncha), did not show such an elevation. Measures of gene expression via RNAseq and qRT-PCR showed that

several of the transporter gene duplicates exhibit elevated expression in the bacteriocyte cells housing nutritional symbionts. These findings suggest the potential for symbiosis to drive trends of host genome evolution. Indeed, the nutrient exchange occurring across the bacteriocyte–hemolymph interface suggests that efficient transport should be of the essence. Additional copy numbers of transporter genes, combined with sub- or neo-functionalization, may, thus, be important attributes enabling the functioning of these symbioses.

Mechanisms of symbiont colonization and persistence

The symbiosis between the bobtail squid *Euprymna scolopes* and bioluminescent *Vibrio fischeri* bacteria has been intensively studied for over 25 years and has become one of the preeminent model systems for understanding host–microbe interactions (Ruby 1996; Nyholm & McFall-Ngai 2004). One of the outstanding questions in this model system has been how the bacterial symbionts are able to persist in their hosts and outcompete non-native bacteria from the surrounding seawater. Koch *et al.* (this issue) convincingly show how important bioluminescence is for long-term persistence. Advances in their ability to raise the squid in aquaria beyond the first week after hatching allowed the authors for the first time to examine mechanisms of long-term symbiont colonization and persistence in mature hosts. Experiments with *V. fischeri* strains defective in light production revealed that these mutants were able to colonize the squid, but were then selectively eliminated both in the presence and absence of wild-type (WT) symbionts. Similarly dramatic examples of sanctioning against so-called cheaters have been described in legume–rhizobia symbioses and plant–mycorrhizal associations (Kiers *et al.* 2003, 2011) and are believed to be the result of an evolutionary selection for mutualism called ‘partner choice’. Little is known about cheating and sanctioning in other symbioses, but such research will be essential for identifying the exquisitely selective and active processes that insure maintenance and persistence in host–microbe associations.

While *V. fischeri* live as extracellular symbionts, a number of other symbionts exist intracellularly. Thus, understanding how symbionts are able to persist in host cells and tissues is a key question in host–microbe associations and is particularly hard to answer in symbioses that cannot be easily cultivated or genetically modified like the *V. fischeri* system. Nguyen *et al.* (this issue) circumvented this problem in the sponge symbiosis, using recombinantly modified *Escherichia coli* to examine how ankyrin-repeat proteins (ARPs), encoded by the genomes of sponge-associated bacteria, modulate phagocytosis in cultivable amoeba cells. ARPs mediate protein–protein interactions and are commonly found in eukaryotes, but are now known to also occur in symbiotic and pathogenic bacteria where they play a role in host infection and intracellular survival (McGraw & O’Neill 2004; Al-Khodori *et al.* 2010). So interestingly, after heterologously expressing ARPs from a gammaproteobacterial sponge symbiont in *E. coli*,

Nguyen *et al.* found that these prevented phagocytic digestion by amoebal cells, leading to an accumulation of *E. coli* in the phagosomes of the amoeba. This study is the first to use such an experimental approach in sponges and may well pave the way for further hypothesis driven research on host–microbe interactions if future studies validate that amoebae are good surrogate models for sponges, or even better, other invertebrate host cells.

Conclusions

The discoveries put forth by the articles in this issue advance our understanding of many symbiotic systems and subdisciplines, while helping to show the way forward for the immediate future across the field at large (see Box 1). These studies have drawn from a range of methodologies, but all incorporate molecular tools in significant ways, via transgenics or DNA/RNA sequencing at single-locus or genome-/transcriptome-level scales. The increased adoption of full-scale ‘omics’ approaches—on display in a few articles here—promises an imminent transformation of the symbiosis field, enabling fine-scale resolution of community composition and function from the same sequence datasets. Decreasing costs of sequencing and increasing analytical infrastructure will both help towards this end.

In closing, we note that many of the studies here have been built from a rich conceptual and methodological groundwork laid by over two centuries of symbiosis research. Indeed, most eukaryotes are now recognized as collections of host and symbiotic microbial cells, a concept that redefines the notion of the individual. The fact that humans too are also ‘not alone’ has taken the media and medical field by storm in recent years, although it comes as little surprise to most students of symbiosis. Yet the symbiosis world owes a great deal to research from the human realm, as the rapid development of sequencing and bioinformatics technologies, spurred by the demands of the human genome and microbiome initiatives, has enabled much of the cutting-edge work performed on symbiosis today. It is thus clear, in our opinion, that the continued support for microbiome research in humans and beyond will be integral to the understanding of our species and of much of life itself.

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