Functional Ecology

BRITISH ECOLOGICAL SOCIETY

Functional Ecology 2016, **30**, 1904–1916 doi: 10.1111/1365-2435.12659

ADVANCES AND CHALLENGES IN THE STUDY OF ECOLOGICAL NETWORKS

Merging DNA metabarcoding and ecological network analysis to understand and build resilient terrestrial ecosystems

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Summary

1. Significant advances in both mathematical and molecular approaches in ecology offer unprecedented opportunities to describe and understand ecosystem functioning. Ecological networks describe interactions between species, the underlying structure of communities and the function and stability of ecosystems. They provide the ability to assess the robustness of complex ecological communities to species loss, as well as a novel way of guiding restoration. However, empirically quantifying the interactions between entire communities remains a significant challenge.

2. Concomitantly, advances in DNA sequencing technologies are resolving previously intractable questions in functional and taxonomic biodiversity and provide enormous potential to determine hitherto difficult to observe species interactions. Combining DNA metabarcoding approaches with ecological network analysis presents important new opportunities for understanding large-scale ecological and evolutionary processes, as well as providing powerful tools for building ecosystems that are resilient to environmental change.

3. We propose a novel 'nested tagging' metabarcoding approach for the rapid construction of large, phylogenetically structured species-interaction networks. Taking tree–insect–parasitoid ecological networks as an illustration, we show how measures of network robustness, constructed using DNA metabarcoding, can be used to determine the consequences of tree species loss within forests, and forest habitat loss within wider landscapes. By determining which species and habitats are important to network integrity, we propose new directions for forest management.

4. Merging metabarcoding with ecological network analysis provides a revolutionary opportunity to construct some of the largest, phylogenetically structured species-interaction networks to date, providing new ways to: (i) monitor biodiversity and ecosystem functioning; (ii) assess the robustness of interacting communities to species loss; and (iii) build ecosystems that are more resilient to environmental change.

Key-words: food webs, forestry, host–parasitoid interactions, invasive species, next-generation sequencing

Introduction

The past decade has seen significant advances in the theoretical understanding, construction, visualization and analysis of complex species interactions networks (Ings *et al.* 2009; Fontaine *et al.* 2011; Kéfi *et al.* 2012). Ecological networks describe the interactions between species; and metrics can be used to characterize their structure, complexity and stability. This provides a framework for understanding species' ecological roles and the mechanisms through which biodiversity influences ecosystem function

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(Thompson *et al*. 2012). Furthermore, they can be used to quantify the effects of human activities (Tylianakis *et al*. 2008), with promising novel applications for nature conservation (Kaiser-Bunbury & Blüthgen 2015) and restoration (Montoya, Rogers & Memmott 2012). To date, however, it has been difficult to characterize the structure of most species-rich ecosystems due to sampling, technical and/or logistical constraints (e.g. Gibson *et al*. 2011). Hence, although conceptual frameworks for studying much more complex networks exist (Fontaine *et al*. 2011), most ecological network studies have tended to focus either on simple, qualitative food webs within and between ecosystems (e.g. Dunne, Williams & Martinez 2002a), or on quantitative interactions within bipartite networks (e.g. host–parasitoid food webs, Tylianakis, Tscharntke & Lewis 2007).

Pocock, Evans & Memmott (2012) were some of the first to construct and analyse a 'network of ecological networks', providing new analytical tools for understanding both the consequences of species extinctions across multiple animals groups, and the potential for ecological restoration within terrestrial ecosystems. These networks were constructed using 'traditional' construction approaches relying on field observations or rearing specimens followed by morphological identification by taxonomists (we use the term 'traditional' throughout to contrast with molecular approaches for network construction from field-collected samples). Although species interactions were highly resolved and well quantified for many of the subnetworks (e.g. plant–insect pollinators), others were potentially subject to bias (e.g. plant–leafminer–parasitoids) because of the limitations of taxonomically selective rearing success and the reliance on accurate morphological identification. Moreover, the construction of such networks is labour-intensive and, unless sampling efficiency can be increased and biases reduced, it is unlikely that these approaches will be used more widely. Thus, in order to construct and analyse multiple, highly resolved ecological networks in an efficient manner, new methods are needed, particularly for poorly studied species and/or interactions that are difficult to observe, such as host–parasitoid food webs (Hrček & Godfray 2015).

Concomitant with advances in network theory and analysis has been the development of powerful DNA-based approaches for individual and community characterization (see Box 1 for a glossary of commonly used terms). Recently, DNA metabarcoding (which involves parallel sequencing of whole communities often obtained as bulk tissue samples, e.g. from arthropod traps) has been found to be taxonomically more comprehensive and many times quicker to produce than traditional monitoring methods (Ji *et al*. 2013). As identifications are genetic rather than morphological, it is less reliant upon taxonomic expertise, making it especially valuable for sampling poorly known taxa and ecosystems. Also, DNA-based approaches can be used to identify remnant DNA shed into the environment (often referred to as environmental DNA or eDNA), allowing the characterization of communities without the presence of whole organisms (e.g. Derocles *et al*. 2015). Although there are still technical issues to overcome (Cristescu 2014), community metabarcoding and eDNA are fast becoming important tools in biodiversity monitoring and conservation (Ji *et al*. 2013; Thomsen & Willerslev 2015). Moreover, they provide unprecedented opportunities to aid in the construction and analysis of ecological networks, particularly if species interactions can also be determined.

One system where DNA-based approaches to construct ecological networks could be fruitfully applied is forests. Forest ecosystems hold a large proportion of global biodiversity and terrestrial carbon stocks, and are key to understanding the mechanisms and management of human-induced global change (Coomes, Burslem & Simonson 2014). Forests have been the subject of pioneering studies of both ecological networks (e.g. Morris, Lewis & Godfray 2004; Tylianakis, Tscharntke & Lewis 2007) and the use of molecular tools in creating networks (e.g. plant–fungi networks; Bennett *et al*. 2013; Toju *et al*. 2014). From a management perspective, the resilience of forests (i.e. the

Box 1. A glossary of terms commonly used in the metabarcoding literature. As this is a rapidly developing field, there is still some ambiguity in the use of terminology as well as additional terms. For a comprehensive list, see Cristescu (2014).

1. Sanger sequencing: also known as dye-terminator sequencing. A polymerase chain reaction-based sequencing technique that provides a DNA sequence for a single locus for a single individual per analysis.
2. Parallel sequencing: also known as next-generation sequencing. A range of sequencing technologies that provide DNA sequences for many DNA fragments simultaneously allowing researchers to analyse many loci or individuals per analysis.
3. Barcoding: the use of one or more genetic loci to identify or detect species. The locus chosen varies by group of organism and sequencing technology used.
4. Metabarcoding: Parallel sequencing of bulk DNA mixtures to detect the species present in whole communities. This may use bulk tissue samples (e.g. kick samples or malaise trap samples) or may use eDNA .
5. Metagenomics: Analysis of whole genomes (currently only mitochondrial genomes) reconstructed from bulk DNA mixtures.
6. Environmental DNA (eDNA): DNA shed into the environment by organisms through a variety of means. This DNA is often of poor quality and presents as short fragments which have been degraded through biological and chemical processes in the environment. Environmental DNA is a term separate to the sequencing technology used, and it is possible to find examples where eDNA has been used with both barcoding and metabarcoding approaches.

capacity of a forest to withstand and absorb external pressures and return, over time, to its pre-disturbance state) is of major policy relevance (Thompson *et al.* 2009), especially in the face of invasive species, pathogens and climate change (Kurz *et al.* 2008). To address these management challenges requires a comprehensive understanding of how species in forest communities interact, how this is related to ecosystem functioning and how they respond to environmental change.

Here, we describe recent advances in ecological network analysis (ENA) and briefly examine how DNA-based methods are increasingly used to quantify species interactions, contrasting the merits of these approaches with traditional approaches (Fig. 1a–d). We discuss how the construction of large, highly resolved, phylogenetically structured ecological networks (Fig. 1e) can be analysed and modelled with ENA (Fig. 1f) and how this can inform the management of ecosystems (Fig. 1g), such as determining the ecological consequences of tree loss and building ecosystem resilience in the face of environmental change. Throughout, our aim is to highlight how molecular biologists can effectively work with network ecologists and *vice versa*. It is not our intention to provide an exhaustive review of molecular methods or ENA, which can be found elsewhere (e.g. Kéfi *et al.* 2012; Cristescu 2014).

To illustrate our conceptual advances, we use existing species-interaction data gathered from the UK Database of Insects and their Food Plants (DBIF) (Smith & Roy 2008) and the Universal Chalcidoidea Database (Noyes 2015) to construct forest networks. Both of these databases have been collated from the literature and casual observer records. We purposely present these large yet incomplete data sets in order to illustrate inherent biases within many existing species-interaction data bases and to demonstrate the need for metabarcoding as a complementary method for constructing better-resolved ecological networks. Plant–herbivore and herbivore–parasitoid associations were extracted and combined from each data base and filtered to produce lists of unique interactions in R version 3.1.3. We use the R package 'HiveR' (Hanson 2016) to visualize our networks throughout. Although we focus on forest plant–herbivore–parasitoid interactions, by merging ENA with metabarcoding we contend that it will be possible to include a considerably wider range of interactions than is possible with traditional network construction approaches, both across trophic levels and within poorly described ecosystems.

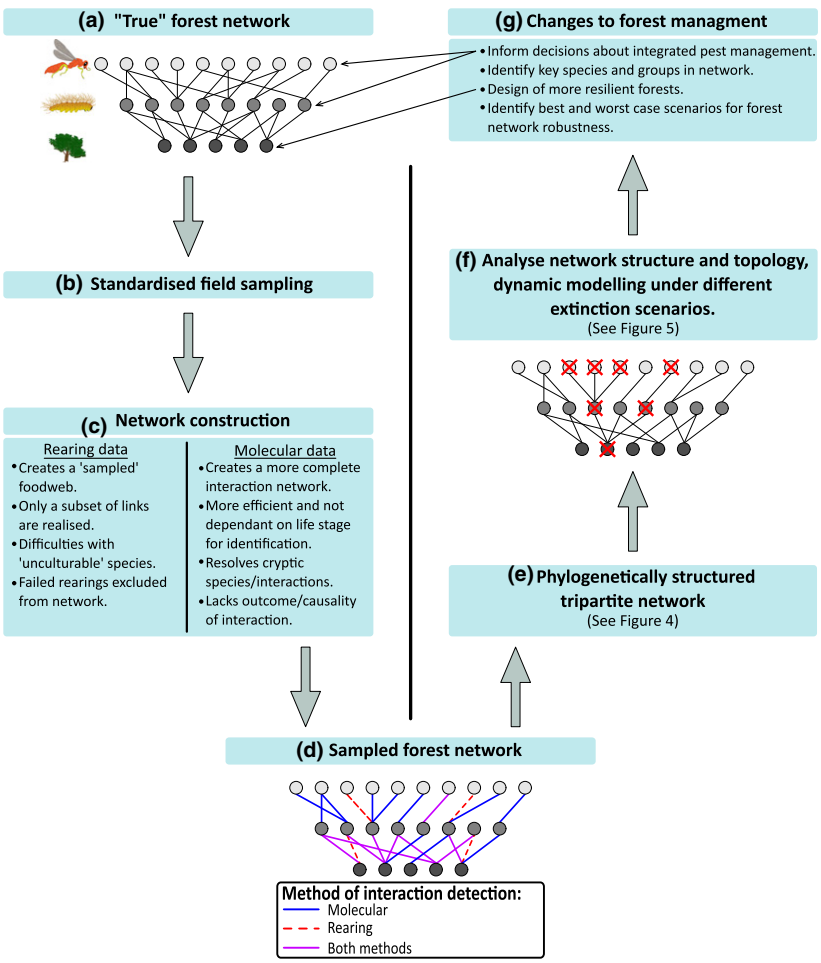

Fig. 1. The steps involved in constructing and analysing large, phylogenetically structured species-interaction networks to inform forest management, here considering a plant–herbivore–parasitoid network, but applicable to any ecological network. In order to create a complete, tripartite network (a), forest plants and arthropods are sampled using standard census techniques (b) and their interactions are determined through traditional identification and rearing, and/or molecular approaches (c), both of which have advantages and disadvantages, but which when combined result in the closest approximation to the 'true' forest network (d). Interactions can be determined using both approaches, but many more (particularly difficult to observe interactions) can be detected using nested tagging metabarcoding and the information generated used to create phylogenetically structured networks (e). The structure and topology of the network can then be analysed and computer modelling used to determine the robustness of the networks to simulated species extinctions (f). Network analysis can be used to inform current forest management, such as targeted pest management, determine the ecological consequences of species loss as well as to suggest a tree species composition that will maximize the robustness of future forests (g).

Advances in ecological network analysis (ENA)

Ecological networks are a powerful framework for assessing ecosystem organization, dynamics, stability and function (Montoya, Pimm & Solé 2006; Bascompte 2009; Thompson *et al*. 2012). Species-interaction data are mostly collected and analysed as: (i) qualitative (un-weighted) ecological networks, indicating the presence of interactions (L, links) between species (S, nodes); (ii) weighted qualitative networks, where the abundance of species across trophic levels and their interactions are determined; or (iii) quantitative networks, where the frequency of interactions between species are determined. Simple measures of network complexity can be calculated, such as link density (L/S) and connectance (L/S^2). Likewise, there are a host of qualitative and quantitative network metrics to describe the network structure, including commonly used measures of consumer-resource asymmetries such as generality (G) and vulnerability (V), and whole system descriptions such as nestedness and modularity (Bersier, Banašek-Richter & Cattin 2002; Olesen *et al*. 2007; Tylianakis, Tscharntke & Lewis 2007; Almeida-Neto *et al*. 2008).

To date, studies have mostly examined bipartite networks such as mutualistic (e.g. plant–pollinator) or antagonistic (e.g. predator–prey) interactions (Pocock, Evans & Memmott 2012). However, comparative studies of ecological network structures across a wider range of network types have as follows: (i) revealed general patterns in how consumer–resource interactions among species are organized (Dunne, Williams & Martinez 2002b; Stouffer *et al*. 2005; Williams & Martinez 2008); (ii) produced successful simple models to characterize such structure (Allesina, Alonso & Pascual 2008); and (iii) supported research on the 'robustness' (a measure of the tolerance of the network to species extinctions) of food webs to species loss (Dunne, Williams & Martinez 2002a; Staniczenko *et al*. 2010).

NETWORK ROBUSTNESS

Of the numerous ecological network attributes, robustness has received particular attention, driven both by advances in the application of computational modelling (Kaiser-Bunbury *et al*. 2010; Staniczenko *et al*. 2010) and by the desire to understand the consequences of biodiversity loss to ecosystem functioning (Pocock, Evans & Memmott 2012). Our understanding of the robustness of networks to species loss has advanced from studies of simple, qualitative bipartite networks (Memmott, Waser & Price 2004), to the investigations of patterns across ecosystems (Srinivasan *et al*. 2007) and to the current quantitative approaches that take into account species abundance (Kaiser-Bunbury *et al*. 2010; Evans, Pocock & Memmott 2013). Classical robustness studies focussed on the consequences of random and non-random biodiversity loss in ecological networks (Dunne, Williams & Martinez 2002a) and are still widely used in ecology, despite the development of more realistic extinction scenarios (Srinivasan *et al*. 2007). Recent approaches incorporate the dynamics of species interactions (rewiring) (Staniczenko *et al*. 2010), examine stochastic coextinction cascades (Vieira & Almeida-Neto 2015) or use a Bayesian analytical framework for dynamic models (Eklöf, Tang & Allesina 2013).

Within forests, network robustness provides clear ways of: (i) predicting the ecological consequences of tree loss (e.g. due to insect pests and disease); (ii) quantifying the overall robustness of forests to sequential species extinction; and (iii) identifying important tree species [i.e. the 'topological keystone species' within the networks (Jordán 2009)]. These analytical approaches are discussed later, but before they can be used it is essential to find ways of efficiently constructing large-scale forest networks. DNA-based methods, in particular metabarcoding, offer unprecedented opportunities to achieve this.

Why use DNA-based methods to construct and analyse ecological networks?

To date, most ecological networks are constructed using non-molecular methods to directly record species interactions whether those interactions are trophic, mutualistic or parasitic. These methods either require field observation of the interactions (e.g. plant–pollinators, Gibson *et al*. 2011), sample collection followed by analysis (e.g. Carnicer, Jordano & Melián 2009) or specimen rearing and identification (e.g. insect herbivores and parasitoids, Evans *et al*. 2011). They are almost always very labour-intensive (Hegland *et al*. 2010), prone to sampling biases (Gibson *et al*. 2011) and can miss cryptic species and associated interactions (Derocles *et al*. 2015). DNA-based approaches can be faster, more efficient and taxonomically more comprehensive than traditional approaches. Combining traditional network construction methods with molecular identification approaches will usually result in more complete and highly resolved ecological networks (Wirta *et al*. 2014). However, DNA-based sampling approaches are not without their own challenges and biases.

To illustrate why combining molecular approaches with empirical observations is important, we visualize the known interactions between all British tree genera, herbivores and their associated parasitoids (mostly using traditional methods) in Fig. 2a. Although the network appears highly resolved, it only includes herbivores where a known interaction with a parasitoid has been observed. However, when all tree–herbivore interaction data are included, as shown in Fig. 2b, the network structure changes significantly and it becomes apparent that considerable herbivore–parasitoid data are missing. Thus, conducting network-level analyses using this incomplete data set will give misleading results. For this data base, considerable sampling effort is needed to elucidate any 'missing links', particularly rare interactions. Molecular methods can play a valuable role in overcoming such issues, either through the mass sampling of forest plant and animal communities or through eDNA approaches, both of which can provide

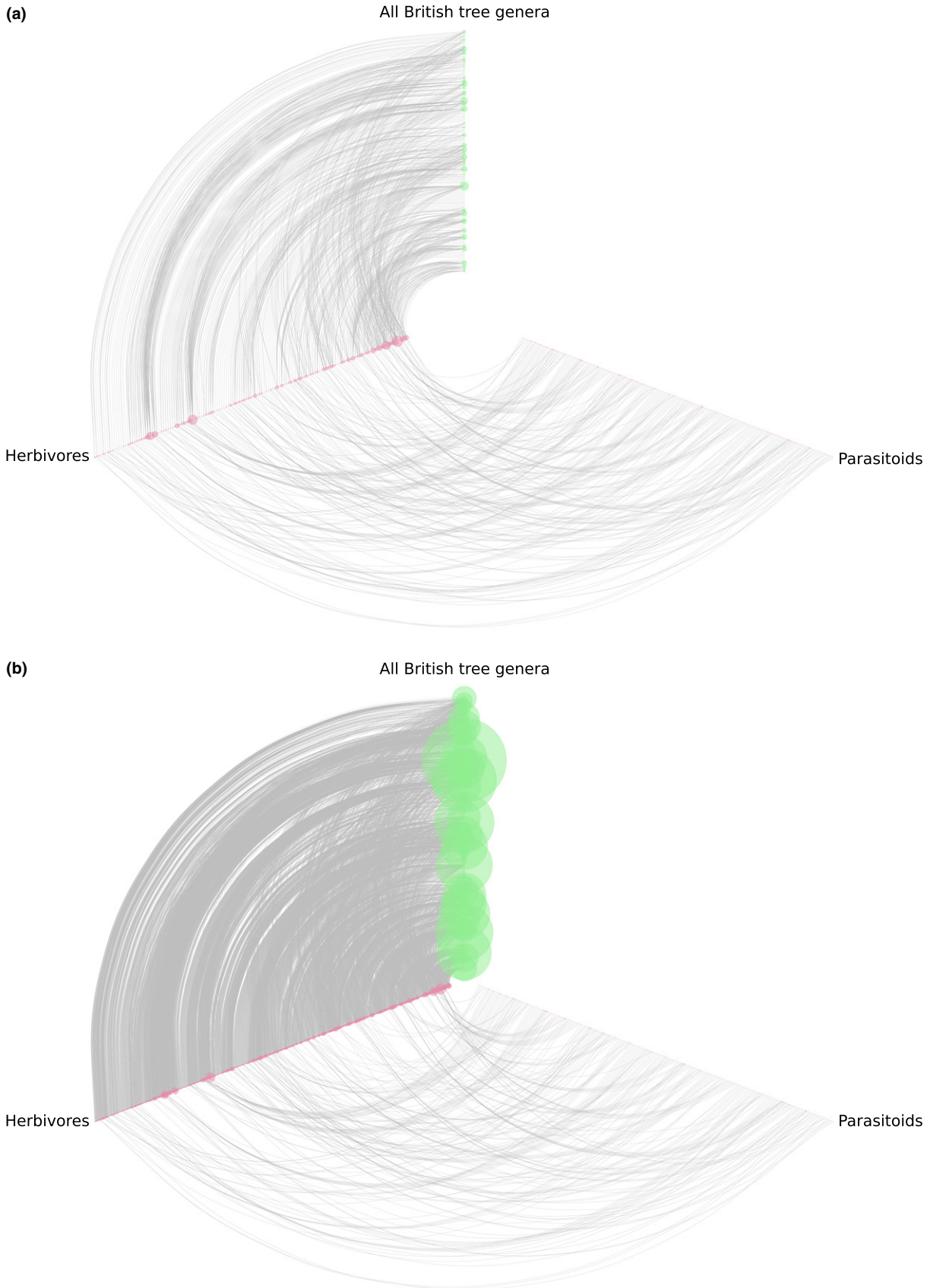

Fig. 2. Tritrophic hive plots of native British tree genera, their herbivores and parasitoids. (a) contains only those herbivore species for which parasitoid interactions have been recorded, while (b) contains all known plant–herbivore interactions. Node sizes are scaled by the number of links connecting to them. An explanation of how this diagram has been created is available in the Supporting Information, Data S1.

high taxonomic resolution. Furthermore, they allow the construction of phylogenetically structured ecological networks, a growing area in network ecology (Elias, Fontaine & van Veen 2013). We briefly examine how molecular approaches have enhanced the ability of ecologists to determine species interactions before describing a novel method to construct ecological networks using metabarcoding, thus overcoming some of the problems associated with traditional network construction methods.

How molecular approaches can enhance our ability to determine interactions

OBSERVATION AND MORPHOLOGICAL TECHNIQUES

Traditional methods for constructing species-interaction networks are often time-consuming or require a high level of taxonomic expertise making them impractical for large-scale studies, particularly in parts of the world with poorly described biota. Indeed, even in well-described ecosystems, organisms are often 'lumped' or assigned by 'morphotype' in ecological networks if they cannot be identified to species level by taxonomists (see early networks such as Memmott 1999). To overcome this, some of the traditional methods can be complemented with, or replaced by, DNA-based approaches to identify interactions that are otherwise difficult to detect. Importantly, the throughput of well-designed molecular approaches can lead to data sets considerably larger than those that can be produced by rearing or observation approaches alone. Examples include trophic interactions (Kitson *et al.* 2013; Clare 2014) and host–parasitoid interactions (Derocles *et al.* 2014; Wirta *et al.* 2014). There is, of course, no single molecular approach suitable for all ecological systems or questions, and the DNA-based methods employed are typically tailored to the specific question being addressed.

PCR DIAGNOSTIC APPROACHES

Researchers must first consider whether the diagnostic method should be sequence-based, since although DNA sequence data give most information there can be significant costs associated in terms of both time and money. To avoid sequencing all samples, it is sometimes possible to develop taxonomically diagnostic polymerase chain reaction (PCR) assays. This approach is an individual-level diagnostic tool and not generally appropriate for the analysis of community samples, but it can be both cheap and quick, with a single person typically producing data for ~1000 samples in a few days. Diagnostic PCR-based approaches can be employed when the study system is relatively simple, and all nodes in the network are known in detail *a priori*. Specific primer pairs can be designed for each species, or set of species, which produce a different PCR amplicon size for each primer pair. Species identification is then as simple as separating the PCR products by gel electrophoresis and measuring the size of each band against a size standard to determine which species-specific amplicon it represents. Derocles *et al.* (2014) employed this approach to detect and identify hymenopteran parasitoids of aphids in agroecosystems. A modification of this is to use fluorescently labelled PCR primers and read the fragment sizes on a DNA analyser, a similar method to that used for microsatellite genotyping. This has advantages over the gel electrophoresis approach as the PCR amplicon related to each species can overlap in size provided each primer pair is labelled with a different fluorescent dye. King *et al.* (2011) employed this approach to identify diet in generalist Carabid beetles active in agroecosystems. In general, diagnostic PCR approaches require significant development of comprehensive primer sets matching all species of interest present in the study system, and it is best seen as a complementary development to sequencing approaches rather than as an alternative.

DNA BARCODING BY SANGER SEQUENCING

For study systems where the full range of organisms interacting is not known *a priori*, identification is best performed by sequencing a barcode gene (i.e. a sequence that is unique to each species). For animals, this is usually Cytochrome c oxidase subunit I (COX1), which has an enormous reference data base (Hebert *et al.* 2003); for plants, this is usually Maturase K (matK), large subunit Ribulose-1,5-bisphosphate carboxylase (rbcLa) or Transfer RNA Leucine intron (trnL) (Hollingsworth, Graham & Little 2011); for fungi, this is usually one or more of the ribosomal internal transcribed spacer regions (ITS) (Seifert 2009). The selection of different loci for different groups originates from the availability of primer pairs that amplify successfully across a wide range of species, and the existence of historically differing large data bases of reference sequences to which the researcher's barcode sequences can be compared in order to identify taxa. In addition, for each locus a range of primer pairs often exist. For instance, Folmer *et al.* (1994) and Leray *et al.* (2013) both amplify COX1 but produce different overlapping fragment lengths. Which primer pair is optimal for a given experimental design is dependent on the specific binding affinities for each primer to the genomes of the studied organisms, as well as on the quality of the DNA extraction (e.g. eDNA is typically degraded compared to tissue extracted DNA and will amplify more successfully when using primers that target a smaller region of a barcode gene).

Sanger sequencing has been used to compare networks constructed using molecular detection with those made using traditional rearing of parasitoids from hosts, with molecular techniques identifying many more interactions than seen when rearing (e.g. Wirta *et al.* 2014). This approach is cheap and easy for small numbers of samples and provides long DNA sequences (upwards of 1000 base pairs where primers allow) leading to higher taxonomic resolution in the DNA sequences, but is unsuited to situations where complex mixtures of DNA may be present.

DNA barcoding is a highly optimized methodology, amenable to efficient processing of samples from moderate-sized projects and is now the standard approach to characterizing biological systems. It produces large amounts of taxonomically relevant information and, given a suitable set of reference sequences, can be highly accurate in species identification. However, the ability to scale this approach to larger and more cost-effective projects remains a challenge since both the resources and time required scale linearly. New sequencing technologies are required to address these issues.

MASSIVELY PARALLEL SEQUENCING AND METABARCODING

When dealing with samples which are complicated mixtures of DNA from multiple species, the individual-level approaches described above are very difficult to employ, and it is much more appropriate to use massively parallel sequencing technologies (also called next-generation sequencing, NGS). The most effective approaches in ecological contexts are called 'metabarcoding' (see Box 1) as they involve the amplification of a barcode sequence from a community sample (pooled individuals), followed by NGS. This results in >1 million sequences, thus covering the species in the sample whose barcode sequence was amplified, but requires detailed bioinformatic analysis to determine taxonomic identities. Identification can be made by reference to existing sequence libraries, but the sequence data allow all operational taxonomic units (OTU) to be distinguished, even if its precise taxonomic identity is unknown. This technology, using platforms such as Roche 454, Life Sciences Ion Torrent and Illumina HiSeq/MiSeq, allows many sequences to be read simultaneously, both within and across biological samples. In particular, their parallel nature provides a means to analyse very complicated DNA mixtures previously unsuitable for standard barcoding, such as bulk samples from insect surveys (Ji *et al*. 2013); eDNA in seawater (Thomsen *et al*. 2012); generalist insectivore diets where the gut contents of any individual may contain many different prey items (Krüger *et al*. 2014; Piñol *et al*. 2014); and plant–fungus interactions in which plant roots may interact with many different fungal species simultaneously (Toju *et al*. 2014).

Perhaps one major reason that NGS community sequencing approaches are yet to be more widely adopted in network ecology is the absence of interaction data. Although it is possible to determine the list of species present in a biological sample (this may be several thousand for some habitats) explicit interaction data between those species is lacking (although it can sometimes be inferred, e.g. Vacher *et al*. 2016). Additionally, many network ecology approaches have relatively simple DNA mixtures present in each sample (a single host–parasite interaction, for example) but a large number of samples would be required to create a representative network. As individual NGS analysis of each sample would be prohibitively expensive, and the more efficient approach of pooling samples into a single cost-effective NGS run would remove the ability to identify interactions, an intermediate method is required in order to obtain both species and interaction data for network construction.

A 'nested tagging' method for creating highly resolved ecological networks with NGS

The challenges of cost efficiency in NGS yet retaining information on interactions can be overcome by advances in sample 'tagging' protocols [some varieties of which have been used for almost a decade e.g. Binladen *et al*. (2007)]. We propose a 'nested tagging' extension of the standard Illumina 16S metabarcoding protocol (Illumina 2011), that fully exploits the capacity of NGS sequencing while retaining the individual-level data most valuable to ecologists (Kitson *et al*. 2016). We describe below, by reference to forest systems, that this approach could be well suited to constructing ecological networks because it will help to resolve the incomplete or missing tree–insect–parasitoid interactions (Fig. 1b) and provide additional information to construct phylogenetically structured networks.

The DNA amplification and nested tagging process is described in Fig. 3. 'Tagging' refers to the addition to the PCR primer of a characteristic DNA sequence not present in the genome being identified. We may include, for example, a unique 4–10 nucleotide sequence at the end of our PCR primer, using a different sequence for each set of primers (Binladen *et al*. 2007). Each PCR amplification can therefore associate a unique sequence with whichever sample was being amplified, and this can be tracked through to the final analysis to identify which sequences came from which individual. The challenge here is to scale this approach, since even a medium sized experiment soon requires thousands of unique primers, which would be both too costly and technically challenging to utilize in the laboratory. The 'nested' approach we describe can reduce the barcode complexity considerably, making large-scale experiments tractable. Individual insects have DNA extracted in 96-well plates, and the COX1 barcode locus is amplified using universal primers. Any of the published primer pairs COX1 would be suitable, provided they produce a PCR amplicon across a wide range of taxa. To each primer, we add a first set of molecular identification (MID) tags, the Illumina sequencing primer and a bridge sequence, so that these elements are incorporated into the PCR product. For each plate, twelve separate forward primers and eight separate reverse primers (differing only by the MID tag) are used. Each column of wells has a different forward primer, and each row a different reverse primer, which when combined gives 96 uniquely MID-tagged PCR products within each plate. Every plate is amplified using the same 96 primer combinations so that MID tag combinations are shared across plates. Each plate is then pooled into its own library of sequences, and each library is re-amplified with another set of primers containing the bridge sequence, a second set of MID tags (this time to

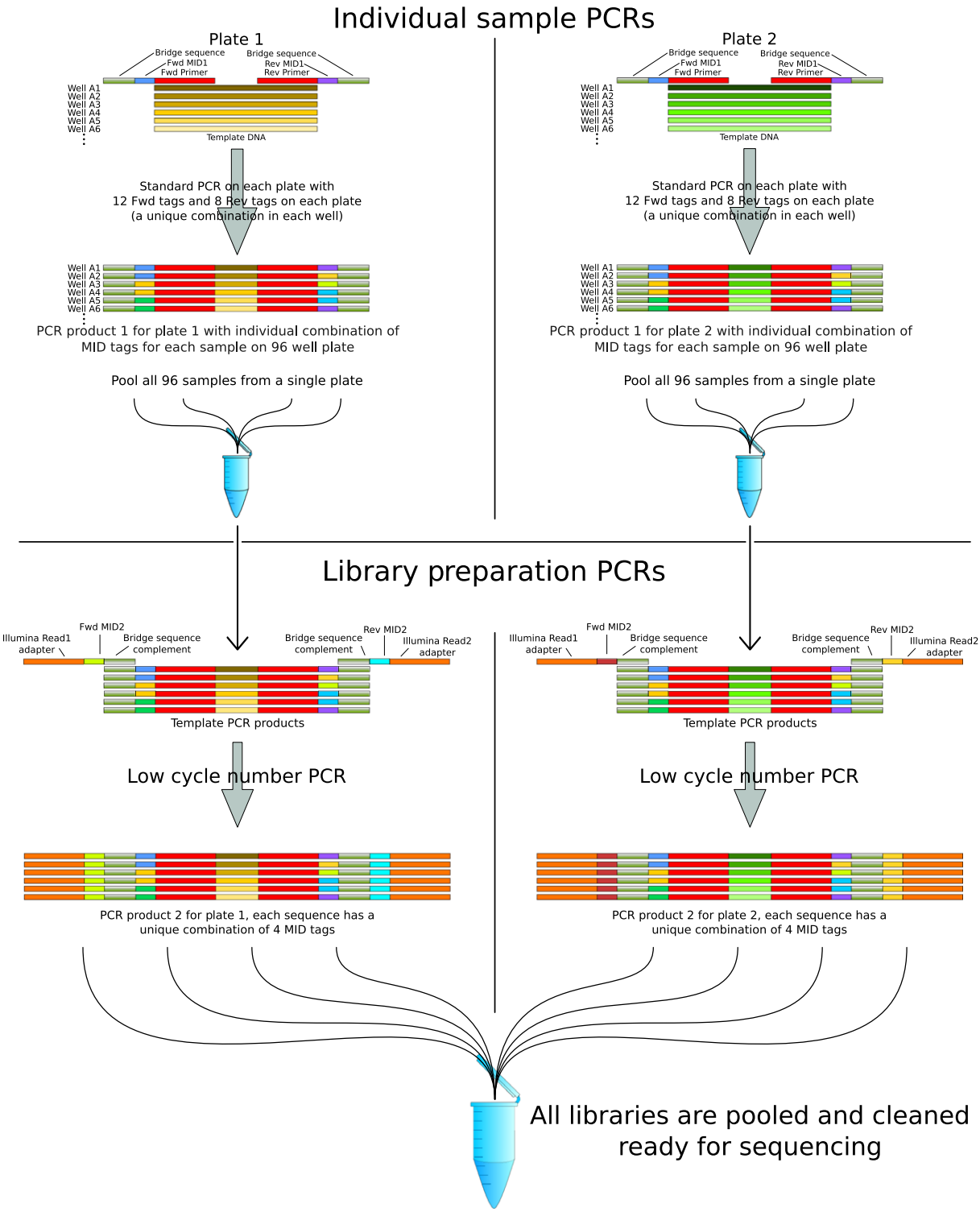

Fig. 3. The tagging and pooling regime required for 'nested tagging' Illumina barcoding. Universal primers with MID tags are used to selectively amplify part of the COX1 barcode region and individually tag each individual on a plate. A PCR-based library preparation protocol is then used to both add MID tags for each plate and add the Illumina plate adapters for sequencing.

identify the plate) and the Illumina adapter sequence for binding to the sequencing flow cell. The result is that each sequence within each library shares the same plate MID tags and, while the individual MID tags are shared across plates, each individual well in the study has its own unique combination of four MID tags, allowing individuals to be reconstructed from the reads.

The nested tagging approach could significantly help in the construction of networks of ecological networks within forests. If biological samples are tagged and pooled for

nested metabarcoding, then information on the tree species (and individual) interactions can be obtained. If a range of tree species (and other woodland plants) are sampled, then the interactions between trees and other organisms (and across trophic levels) can be analysed, ranging from large-scale food webs to more subtle effects on networks, such as intracellular parasites, diseases and linkages between herbivore and host genotypes.

CHALLENGES IN USING MOLECULAR TOOLS FOR ECOLOGICAL NETWORK ANALYSIS

The most urgent research need for metabarcoding is to promote best common practices for data analysis (Cristescu 2014). Metabarcoding studies provide biodiversity estimates that are highly dependent on the resolution of the marker used, the quality of the sequence libraries, and the parameters used in bioinformatics pipelines. Currently, metabarcoding and nested tagging metabarcoding (as described above) is limited to sequencing approximately 600 bp or less which can limit the level to which taxonomic assignments can be made (e.g. Taberlet *et al.* 2006). Although analysis allows OTUs to be distinguished even when the DNA sequence cannot be assigned to a named species, these OTUs are not easily reconcilable across sites or studies, thus making it difficult to draw species-level conclusions from the data. However, in most contexts, we suggest that, even with suboptimal locus choice, the resolution achievable for many taxonomic groups would still be superior compared with assigning specimens to morphospecies based on external appearance.

One specific advantage of sequence data is that not only can species (or OTUs) be identified, but that their relatedness can be ascertained via phylogenetic analysis of the sequence data. However, shorter loci can make phylogenetic inferences among the sampled species less reliable. To circumvent these problems and provide more robust estimates of the relatedness of taxa in the samples, it is possible to take a phylogenetic approach to taxon identification. Programs such as pplacer (Matsen, Kodner & Armbrust 2010) and RAxML-EPA (Caporaso *et al.* 2010; Berger, Krompass & Stamatakis 2011) build a phylogenetic tree that includes longer sequences from related species sourced from GenBank, and to estimate relationships and identifications among the unknown taxa.

Application of ecological network analysis (ENA) and metabarcoding to forest ecosystems

UNDERSTANDING THE STRUCTURE OF FOREST ECOLOGICAL NETWORKS AND THEIR RESPONSE TO ENVIRONMENTAL CHANGE

Despite the importance of forests for global biodiversity, species interactions within them are still poorly understood. However, ENA has been used in several ways in forest systems to show, for example: how forest insects can interact through shared natural enemies via apparent competition (Morris, Lewis & Godfray 2004) and in the face of changing environmental conditions (Staab, Blüthgen & Klein 2014); that logging old-growth forest reduces the redundancy of networks of birds feeding on fruits (Albrecht *et al.* 2013); and how modifying the forest structure impacts more upon network structure than species assemblages (Tylianakis, Tscharntke & Lewis 2007). These examples highlight how ENA can be used to better understand ecological and evolutionary processes within forests, as well as its potential for determining the impacts of environmental change on ecosystem functioning. The increased efficiency granted by nested tagging metabarcoding will make it more tractable to construct and analyse large-scale, highly resolved forest networks.

INCORPORATING PHYLOGENETIC INFORMATION INTO ECOLOGICAL NETWORK ANALYSIS

Combining phylogenetic information with ENA can make a significant contribution to our understanding of the structure and fate of species-rich communities (Vázquez, Chacoff & Cagnolo 2009; Elias, Fontaine & van Veen 2013; Rafferty & Ives 2013). Figure 4 shows how nested tagging metabarcoding provides the data necessary to construct phylogenetically structured ecological networks. To date, most species-interaction data generated using traditional field observations and insect rearing has been organized in a manner similar to that shown in Fig. 4a. Here, the species-interaction matrices represent the supposed frequency of interaction between a subset of trees, herbivores and parasitoids for illustrative purposes. By adding the phylogenies of the trees, herbivores and parasitoids to the matrices (Fig. 4b), it is possible to investigate the presence of phylogenetic signals in the ecological networks and variation within and between trophic levels (Elias, Fontaine & van Veen 2013). Merging DNA metabarcoding with ENA has considerable potential for phylogenetic trait-based analyses (Rafferty & Ives 2013), understanding co-evolutionary interactions (Guimarães, Jordano & Thompson 2011) and coextinction cascades of related species (Rezende *et al.* 2007).

EXAMINING THE ROBUSTNESS OF FOREST NETWORKS AND IDENTIFYING KEY TREE SPECIES

In order to understand the cascading effects of tree extinction on biodiversity, for example as a result of disease (Mitchell *et al.* 2014) or invasive insects (Handley *et al.* 2011), assessing the robustness of forest networks is a promising area for future research. We exemplify this with a network of trees (the eight most frequently occurring genera in DBIF), insect herbivores and parasitoids (Fig. 5a). The insects are directly and indirectly connected through shared tree species, which can be removed sequentially either randomly (Fig. 5b and c) or through pre-defined criteria. One useful criterion would be the phylogenetic relatedness of trees or insects, such as natu-

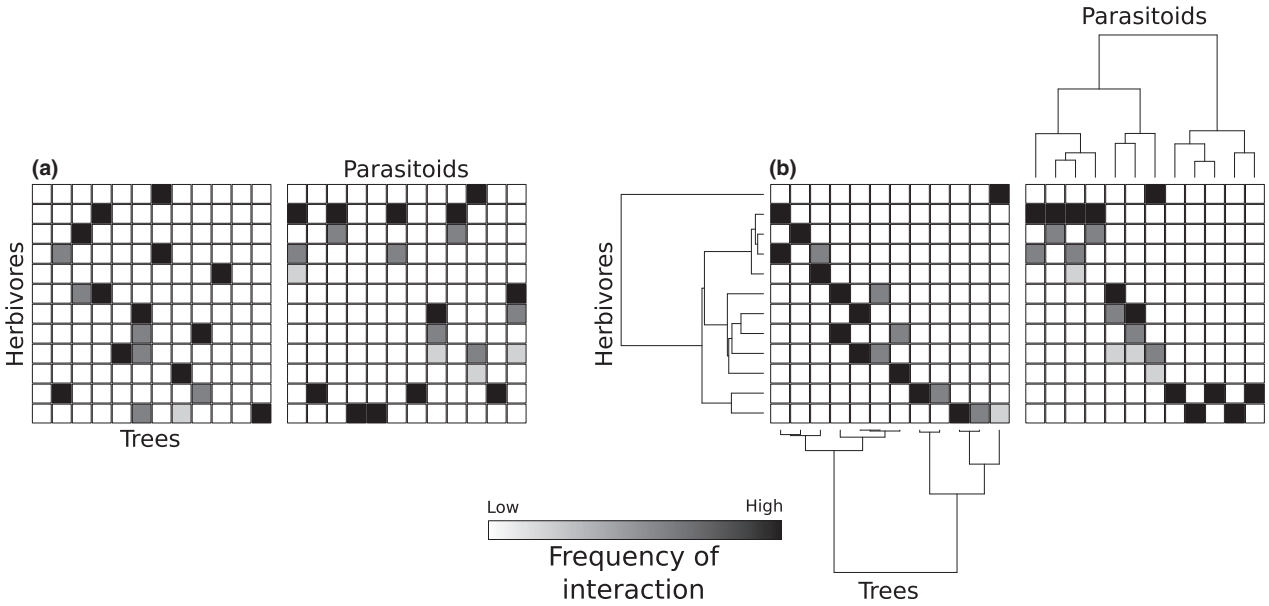

Fig. 4. 'Nested tagging' metabarcoding provides additional data allowing ecological networks to be phylogenetically structured. For illustrative purposes, (a) shows the supposed tree–herbivore and herbivore–parasitoid interactions based on traditional field observations and insect rearing. The frequency of interaction between species is shown by shading, the darker the shading the higher the frequency. By adding the hypothetical phylogenies of the trees, herbivores and parasitoids to the matrices (b), it is possible to investigate the presence of phylogenetic signals in the ecological networks and variation within and between trophic levels (see Elias, Fontaine & van Veen 2013, e.g. across 4 trophic levels). Such information can be used to determine extinction scenarios in robustness analyses.

rally obtained via the nested tagging approach to determine interactions, which is useful to forest managers when considering shared susceptibility of a taxonomically related group of species to a disease or pest. The robustness of the tripartite network (Fig. 5d) can be calculated by recording: (i) the number of herbivore secondary extinctions as a result of sequential tree loss; and (ii) the subsequent number of parasitoid secondary extinctions as a result of herbivore loss (as per Pocock, Evans & Memmott 2012). In this example, the random sequential loss of tree species has little impact on the network at first as many animals have shared hosts, but as more tree species are lost the number of secondary extinctions accelerates. Robustness analysis can be developed further to determine the relative importance of species within the networks, for example their contribution to network robustness (Pocock, Evans & Memmott 2012) thus complementing structural measures of species importance in networks (Jordán 2009).

Robustness has a range of potential applications for forest management. First, if the robustness of the networks of trees and species in dependant guilds (e.g. herbivores and epiphytes) varies considerably between the different guilds, it may be possible to select sensitive groups for conservation effort and assessment as bioindicators. Secondly, if the robustness of animal groups is found to covary, targeting specific guilds for management might have cascading benefits. Thirdly, if some tree species are discovered to be disproportionately important in the network of networks, these trees could be investigated further for building more resilient forests or for planning restoration. This information could also inform impact assessments and the cost/benefit analyses used to determine whether management of pests and diseases is justified. Furthermore, the importance of a tree species in an ecological network (i.e. taking indirect as well as direct interactions into account) could provide one indication of its non-market value.

DETERMINING THE IMPORTANCE OF FORESTS AT THE LANDSCAPE SCALE

Recently, network robustness was developed further to model the cascading effects of habitat loss via plant extinctions on animal groups (Evans, Pocock & Memmott 2013), representing a new method to examine the relative importance of different habitats, including forests, at the landscape scale. This study developed the use of a genetic algorithm (GA; which is an efficient way of searching for global optima) to determine the least-serious and the worst-case habitat loss permutations of extinction sequences [see also (Allesina & Pascual 2009)].

FOREST CONSERVATION AND RESTORATION

Forest managers and conservation practitioners require indicators to monitor and assess management effectiveness and validate conservation goals. Kaiser-Bunbury & Blüthgen (2015) present a framework for network analysis to be incorporated into conservation management with an implementation pathway that outlines the stages required to successfully embed a network approach. Other emerging perspectives in the restoration of biodiversity-based

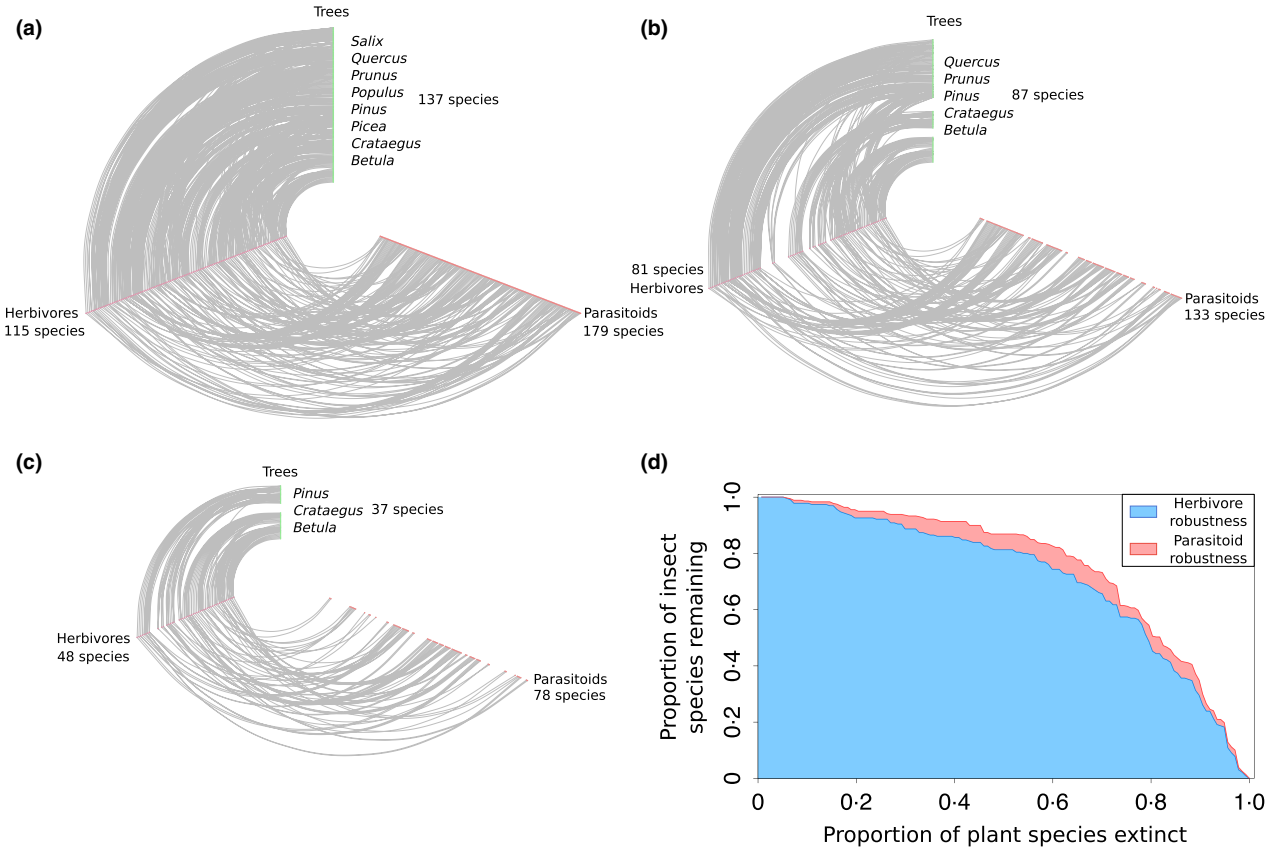

Fig. 5. Tree loss has consequences across trophic levels. Tree genera have been selected to include the 8 most frequently featured in the DBIF data base showing: (a) all interactions between the selected tree genera and their herbivores with known parasitoids; (b) and (c) successive random tree extinction; and (d) the cascading extinctions across trophic levels. An explanation of how this diagram has been created is available in the Supporting Information, Data S1.

ecosystem services using ecological networks have been proposed (Montoya, Rogers & Memmott 2012). For example, a recent study by Ribeiro da Silva *et al.* (2015) demonstrated how ecological networks can be used as an indicator of the restoration success of Atlantic rain forests. With increasing threats to tree health via invasive species, diseases and climate change, we believe that combining metabarcoding with ENA will provide forest managers with practical information to potentially enhance resilience. The additional phylogenetic data obtained from metabarcoding will provide important information about how trees with differing evolutionary histories respond to a range of biotic and abiotic stresses (e.g. Robinson *et al.* 2015). Considering the future of forests, the information from this combined approach will support forest managers in developing much-needed responses based on adaptation, migration or extirpation (Aitken *et al.* 2008).

Conclusion

Combined advances in metabarcoding, complexity science and 'Big Data' provide unprecedented opportunities to create some of the largest, highly resolved and phylogenetically structured ecological networks to date. Metabarcoding is resolving previously intractable questions in functional and taxonomic biodiversity, and there is a growing interest in how to infer species interactions based on functional traits, phylogenies and geography (Morales-Castilla *et al.* 2015). By merging nested tagging metabarcoding with ENA, interaction data can be retained. Within forests, it can provide better-resolved species-interaction networks and allows a novel way of determining robustness, the importance of tree species to network integrity and ultimately forest species composition to maximize resilience (Oliver *et al.* 2015). The combined approaches are applicable to other ecosystems and can provide a new way to better understand, predict and manage complex species interactions in a changing world.

Acknowledgements

We thank Timothée Poisot, Sonia Kefi and Daniel Stouffer for comments on an early draft and the two anonymous reviewers for their helpful input. D. M. Evans and J. J. N. Kitson cowrote as joint-first authors the first draft of the manuscript, and all authors contributed substantially to revisions.

Data accessibility

No data are archived for this manuscript. All data are publicly available through the Database of Insects and their Food Plants (Smith & Roy 2008) http://www.brc.ac.uk/dbif/ and the Universal Chalcidoidea Database (Noyes 2015).

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Received 26 August 2015; accepted 29 February 2016
Handling Editor: Timothée Poisot

Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Data S1. Data acquisition for network plotting.