EXTENDED SPOTLIGHT:

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

Evans, Darren M.[a,b], Kitson, James J. N.*[b], Lunt, David H.[b], Straw, Nigel A.[c] & Pocock, Michael. J. O.[d]

[a] School of Biology, Newcastle University, Newcastle upon Tyne, NE1 7RU, United Kingdom

[b] School of Biological, Biomedical and Environmental Sciences, University of Hull, Cottingham Road, Hull, HU6 7RX, United Kingdom

[c] Forest Research, Alice Holt Lodge, Farnham, Surrey, GU10 4LH, United Kingdom

[d] Centre for Ecology & Hydrology, Crowmarsh Gifford, Wallingford, Oxfordshire, OX10 8BB, United Kingdom

*Corresponding author: KitsonJJN@gmail.com

Running headline: Metabarcoding and ecological network analysis

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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.

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

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Introduction

The past decade has seen significant advances in the theoretical understanding, construction, visualisation 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 (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 *et al.* (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

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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 sub-networks (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 characterisation (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, many times quicker to produce than traditional monitoring methods (Ji *et al.* 2013), because 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

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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 *et al.* 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 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 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

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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 datasets in order to illustrate inherent biases within many existing species-interaction databases 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 database and filtered to produce lists of unique interactions in R version 3.1.3. We use the R package 'HiveR' (Hanson 2015) 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.

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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 is 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; Tylianakis *et al.* 2007; Olesen *et al.* 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 *et al.* 2012). However, comparative studies of ecological network structures across a wider range of network types have: a) revealed general patterns in how consumer–resource interactions among species are organized (Dunne, Williams & Martinez 2002b; Stouffer *et al.* 2005; Williams & Martinez 2008); b) produced successful simple models to characterize such structure (Allesina, Alonso &

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160 Pascual 2008); and c) supported research on the 'robustness' (a measure of the tolerance of
161 the network to species extinctions) of food-webs to species loss (Dunne *et al.* 2002a;
162 Staniczenko *et al.* 2010).
163
164 *Network robustness*
165 Of the numerous ecological network attributes, robustness has received particular attention,
166 driven both by advances in the application of computational modelling (Kaiser-Bunbury *et al.*
167 2010; Staniczenko *et al.* 2010) the desire to understand the consequences of biodiversity loss to
168 ecosystem functioning (Pocock *et al.* 2012). Our understanding of the robustness of networks to
169 species loss has advanced from studies of simple, qualitative bipartite networks (Memmott,
170 Waser & Price 2004), to investigations of patterns across ecosystems (Srinivasan *et al.* 2007)
171 and to current quantitative approaches that take into account species abundance (Kaiser-
172 Bunbury *et al.* 2010; Evans, Pocock & Memmott 2013). Classical robustness studies focussed
173 on the consequences of random and non-random biodiversity loss in ecological networks
174 (Dunne *et al.* 2002a) and are still widely used in ecology, despite the development of more
175 realistic extinction scenarios (Srinivasan *et al.* 2007). Recent approaches incorporate the
176 dynamics of species-interactions (rewiring) (Staniczenko *et al.* 2010), examine stochastic
177 coextinction cascades (Vieira & Almeida-Neto 2015) or use a Bayesian analytical framework for
178 dynamic models (Eklöf, Tang & Allesina 2013).
179
180 Within forests, network robustness provides clear ways of: i) predicted the ecological
181 consequences of tree loss (for example due to insect pests and disease); ii) quantifying the

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182 overall robustness of forests to sequential species extinction; and iii) identifying important tree
183 species (i.e. the 'topological keystone species' within the networks (Jordán 2009)). These
184 analytical approaches are discussed later, but before they can be used it is essential to find
185 ways of efficiently constructing large-scale forest networks. DNA-based methods, in particular
186 metabarcoding, offer unprecedented opportunities to achieve this.
187
188 Why use DNA-based methods to construct and analyse ecological networks?
189 To date, most ecological networks are constructed using non-molecular methods to directly
190 record species interactions whether those interactions are trophic, mutualistic or parasitic.
191 These methods either require field observation of the interactions (e.g. plant-pollinators, Gibson
192 *et al.* 2011), sample collection followed by analysis (e.g. Carnicer, Jordano & Melián 2009) or
193 specimen rearing and identification (e.g. insect herbivores and parasitoids, Evans *et al.* 2011).
194 They are almost always very labour intensive (Hegland *et al.* 2010), prone to sampling biases
195 (Gibson *et al.* 2011) and can miss cryptic species and associated interactions (Derocles *et al.*
196 2015). DNA-based approaches can be faster, more efficient and taxonomically more
197 comprehensive than traditional approaches. Combining traditional network construction
198 methods with molecular identification approaches will usually result in more complete and
199 highly-resolved ecological networks (Wirta *et al.* 2014). However, DNA-based sampling
200 approaches are not without their own challenges and biases (see below).
201
202 To illustrate why combining molecular approaches with empirical observations is important, we
203 visualize the known interactions between all British tree genera, herbivores and their associated

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parasitoids (mostly using traditional methods) in Figure 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 is included, as shown in Figure 2B, the network structure changes significantly and it becomes apparent that considerable herbivore-parasitoid data is missing. Thus conducting network-level analyses using this incomplete dataset will give misleading results. For this database, 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 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

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226 they cannot be identified to species level by taxonomists (see early networks such as Memmott
227 1999). To overcome this, some of the traditional methods can be complemented with, or
228 replaced by, DNA-based approaches to identify interactions that are otherwise difficult to detect.
229 Importantly, the throughput of well-designed molecular approaches can lead to datasets
230 considerably larger than those that can be produced by rearing or observation approaches
231 alone. Examples include trophic interactions (Kitson *et al.* 2013; Clare 2014) and host-parasitoid
232 interactions (Wirta *et al.* 2014; Derocles *et al.* 2014). There is, of course, no single molecular
233 approach suitable for all ecological systems or questions, and the DNA-based methods
234 employed are typically tailored to the specific question being addressed.
235
236 *PCR diagnostic approaches*
237 Researchers must first consider whether the diagnostic method should be sequence-based,
238 since although DNA sequence data gives most information there can be significant costs
239 associated in terms of both time and money. To avoid sequencing all samples, it is sometimes
240 possible to develop taxonomically diagnostic polymerase chain reaction (PCR) assays. This
241 approach is an individual-level diagnostic tool and not generally appropriate for the analysis of
242 community samples, but it can be both cheap and quick, with a single person typically producing
243 data for ~1000 samples in a few days. Diagnostic PCR based approaches can be employed
244 when the study system is relatively simple and all nodes in the network are known in detail *a*
245 *priori*. Specific primer pairs can be designed for each species, or set of species, which produce
246 a different PCR amplicon size for each primer pair. Species identification is then as simple as
247 separating the PCR products by gel electrophoresis and measuring the size of each band

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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 database (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 databases of

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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 (for example, 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 (see below).

DNA barcoding is a highly optimised methodology, amenable to efficient processing of samples from moderate sized projects and is now the standard approach to characterising 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

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292 the resources and time required scale linearly. New sequencing technologies are required to
293 address these issues.
294
295 *Massively parallel sequencing and metabarcoding*
296 When dealing with samples which are complicated mixtures of DNA from multiple species, the
297 individual-level approaches described above are very difficult to employ, and it is much more
298 appropriate to use massively parallel sequencing technologies (also called next generation
299 sequencing, NGS). The most effective approaches in ecological contexts are called
300 'metabarcoding' (See Box 1) as they involve the amplification of a barcode sequence from a
301 community sample (pooled individuals), followed by NGS. This results in >1 million sequences,
302 thus covering the species in the sample whose barcode sequence was amplified, but requires
303 detailed bioinformatic analysis to determine taxonomic identities. Identification can be made by
304 reference to existing sequence libraries, but the sequence data allows all operational taxonomic
305 units (OTU) to be distinguished, even if its precise taxonomic identity is unknown. This
306 technology, using platforms such as Roche 454, Life Sciences Ion Torrent and Illumina
307 HiSeg/MiSeq, allows many sequences to be read simultaneously, both within and across
308 biological samples. In particular, their parallel nature provides a means to analyse very
309 complicated DNA mixtures previously unsuitable for standard barcoding, such as: bulk samples
310 from insect surveys (Ji *et al.* 2013); eDNA in seawater (Thomsen *et al.* 2012); generalist
311 insectivore diets where the gut contents of any individual may contain many different prey items
312 (Piñol *et al.* 2014; Krüger *et al.* 2014); and plant-fungus interactions in which plant roots may
313 interact with many different fungal species simultaneously (Toju *et al.* 2014).

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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

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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 Figure 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 utilise in the laboratory. The 'nesting' 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

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357 are shared across plates. Each plate is then pooled into its own library of sequences, and each
358 library is re-amplified with another set of primers containing the bridge sequence, a second set
359 of MID tags (this time to identify the plate) and the Illumina adapter sequence for binding to the
360 sequencing flow cell. The result is that each sequence within each library shares the same plate
361 MID tags and, while the individual MID tags are shared across plates, each individual well in the
362 study has its own unique combination of four MID tags, allowing individuals to be reconstructed
363 from the reads.
364
365 The nested tagging approach could significantly help in the construction of networks of
366 ecological networks within forests. If biological samples are tagged and pooled for nested
367 metabarcoding, then information on the tree species (and individual) interactions can be
368 obtained. If a range of tree species (and other woodland plants) are sampled, then the
369 interactions between trees and other organisms (and across trophic levels) can be analysed,
370 ranging from large-scale food-webs to more subtle effects on networks, such as intracellular
371 parasites, diseases and linkages between herbivore and host genotypes.
372
373 *Challenges in using molecular tools for ecological network analysis*
374 The most urgent research need for metabarcoding is to promote best common practices for
375 data analysis (Cristescu 2014). Metabarcoding studies provide biodiversity estimates that are
376 highly dependent on the resolution of the marker used, the quality of the sequence libraries, and
377 the parameters used in bioinformatics pipelines. Currently, metabarcoding and nested tagging
378 metabarcoding (as described above) is limited to sequencing approximately 600bp or less which

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379 can limit the level to which taxonomic assignments can be made (e.g. Taberlet *et al.* 2006).
380 Although analysis allows OTUs to be distinguished even when the DNA sequence cannot be
381 assigned to a named species, these OTUs are not easily reconcilable across sites or studies,
382 thus making it difficult to draw species-level conclusions from the data. However, in most
383 contexts, we suggest that, even with suboptimal locus choice, the resolution achievable for
384 many taxonomic groups would still be superior compared with assigning specimens to
385 morphospecies based on external appearance.
386
387 One specific advantage of sequence data is that not only can species (or OTUs) be identified,
388 but that their relatedness can be ascertained via phylogenetic analysis of the sequence data.
389 However, shorter loci can make phylogenetic inferences among the sampled species less
390 reliable. To circumvent these problems and provide more robust estimates of the relatedness of
391 taxa in the samples it is possible to take a phylogenetic approach to taxon identification.
392 Programs such as pplacer (Matsen, Kodner & Armbrust 2010) and RAxML-EPA (Caporaso *et*
393 *al.* 2010; Berger, Krompass & Stamatakis 2011) build a phylogenetic tree that includes longer
394 sequences from related species sourced from GenBank, and to estimate relationships and
395 identifications among the unknown taxa.
396
397 Application of ecological network analysis (ENA) and metabarcoding to forest
398 ecosystems
399 *Understanding the structure of forest ecological networks and their response to environmental*
400 *change*

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401 Despite the importance of forests for global biodiversity, species-interactions within them are still
402 poorly understood. However, ENA has been used in several ways in forest systems to show, for
403 example: how forest insects can interact through shared natural enemies via apparent
404 competition (Morris *et al.* 2004) and in the face of changing environmental conditions (Staab,
405 Blüthgen & Klein 2014); that logging old-growth forest reduces the redundancy of networks of
406 birds feeding on fruits (Albrecht *et al.* 2013); and how modifying the forest structure impacts
407 more upon network structure than species assemblages (Tylianakis *et al.* 2007). These
408 examples highlight how ENA can be used to better understand ecological and evolutionary
409 processes within forests, as well as its potential for determining the impacts of environmental
410 change on ecosystem functioning. The increased efficiency granted by nested tagging
411 metabarcoding will make it more tractable to construct and analyse large-scale, highly-resolved
412 forest networks.
413
414 *Incorporating phylogenetic information into ecological network analysis*
415 Combining phylogenetic information with ENA can make a significant contribution to our
416 understanding of the structure and fate of species-rich communities (Vázquez, Chacoff &
417 Cagnolo 2009; Elias *et al.* 2013; Rafferty & Ives 2013). Figure 4 shows how nested tagging
418 metabarcoding provides the data necessary to construct phylogenetically structured ecological
419 networks. To date, most species-interaction data generated using traditional field observations
420 and insect rearing has been organised in a manner similar to that shown in Figure 4A. Here the
421 species-interaction matrices represent the supposed frequency of interaction between a subset
422 of trees, herbivores and parasitoids for illustrative purposes. By adding the phylogenies of the

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423 trees, herbivores and parasitoids to the matrices (Fig. 4B), it is possible to investigate the
424 presence of phylogenetic signals in the ecological networks and variation within and between
425 trophic levels (Elias *et al.* 2013). Merging DNA metabarcoding with ENA has considerable
426 potential for phylogenetic trait-based analyses (Rafferty & Ives 2013), understanding
427 coevolutionary interactions (Guimarães, Jordano & Thompson 2011) and coextinction cascades
428 of related species (Rezende *et al.* 2007).
429
430 *Examining the robustness of forest networks and identifying key tree species*
431 In order to understand the cascading effects of tree extinction on biodiversity, for example as a
432 result of disease (Mitchell *et al.* 2014) or invasive insects (Handley *et al.* 2011), assessing the
433 robustness of forest networks is a promising area for future research. We exemplify this with a
434 network of trees (the eight most frequently occurring genera in DBIF), insect herbivores and
435 parasitoids (Fig. 5A). The insects are directly and indirectly connected through shared tree
436 species, which can sequentially be removed either randomly (Figs. 5B and 5C) or through pre-
437 defined criteria. One useful criterion would be the phylogenetic relatedness of trees or insects,
438 such as naturally obtained via the nested tagging approach to determine interactions, which is
439 useful to forest managers when considering shared susceptibility of a taxonomically related
440 group of species to a disease or pest. The robustness of the tripartite network (Fig. 5D) can be
441 calculated by recording: i) the number of herbivore secondary extinctions as a result of
442 sequential tree loss; and ii) the subsequent number of parasitoid secondary extinctions as a
443 result of herbivore loss (as per Pocock *et al.* 2012). In this example, the random sequential loss
444 of tree species has little impact on the network at first as many animals have shared hosts, but

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445 as more tree species are lost the number of secondary extinctions accelerates. Robustness
446 analysis can be developed further to determine the relative importance of species within the
447 networks, for example their contribution to network robustness (Pocock et al. 2012) thus
448 complementing structural measures of species important in networks (Jordan 2009).
449
450 Robustness has a range of potential applications for forest management. First, if the robustness
451 of the networks of trees and species in dependant guilds (e.g. herbivores, epiphytes etc.) varies
452 considerably between the different guilds, it may be possible to select sensitive groups for
453 conservation effort and assessment as bioindicators. Second, if the robustness of animal groups
454 are found to co-vary, targeting specific guilds for management might have cascading benefits.
455 Third, if some tree species are discovered to be disproportionately important in the network of
456 networks, these trees could be investigated further for building more resilient forests or for
457 planning restoration. This information could also inform impact assessments and the
458 cost/benefit analyses used to determine whether management of pests and diseases is justified.
459 Furthermore, the importance of a tree species in an ecological network (i.e taking indirect as
460 well as direct interactions into account) could provide one indication of its non-market value.
461
462 *Determining the importance of forests at the landscape scale*
463 Recently, network robustness was developed further to model the cascading effects of habitat
464 loss via plant extinctions on animal groups (Evans *et al.* 2013), representing a new method to
465 examine the relative importance of different habitats, including forests, at the landscape scale.
466 This study developed the use of a genetic algorithm (GA; which is an efficient way of searching

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467 for global optima) to determine the least-serious and the worst-case habitat loss permutations of
468 extinction sequences (see also (Allesina & Pascual 2009)).
469
470 *Forest conservation and restoration*
471 Forest managers and conservation practitioners require indicators to monitor and assess
472 management effectiveness and validate conservation goals. Kaiser-Bunbury & Blüthgen (2015)
473 present a framework for network analysis to be incorporated into conservation management
474 with an implementation pathway that outlines the stages required to successfully embed a
475 network approach. Other emerging perspectives in the restoration of biodiversity-based
476 ecosystem services using ecological networks have been proposed (Montoya *et al.* 2012). For
477 example, a recent study by Ribeiro da Silva *et al.* (2015) (2015) demonstrated how ecological
478 networks can be used as an indicator of the restoration success of Atlantic rainforests. With
479 increasing threats to tree health via invasive species, diseases and climate change, we believe
480 that combining metabarcoding with ENA will provide forest managers with practical information
481 to potentially enhance resilience. The additional phylogenetic data obtained from metabarcoding
482 will provide important information about how trees with differing evolutionary histories respond
483 to a range of biotic and abiotic stresses (e.g. Robinson *et al.* 2015). Considering the future of
484 forests, the information from this combined approach will support forest managers in developing
485 much-needed responses based on adaptation, migration or extirpation (Aitken *et al.* 2008).
486

487 ## Conclusion

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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 maximise 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.

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Data Accessibility

No data is archived for this manuscript. All data is 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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732

733 Tables

734 Box 1: A glossary of terms commonly used in the metabarcoding literature. As this is a rapidly
735 developing field, there is still some ambiguity in the use of terminology as well as additional
736 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 (see below).
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 present as short fragments which have been degraded through biological and chemical processes in the

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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.

737

738

739 Figure legends

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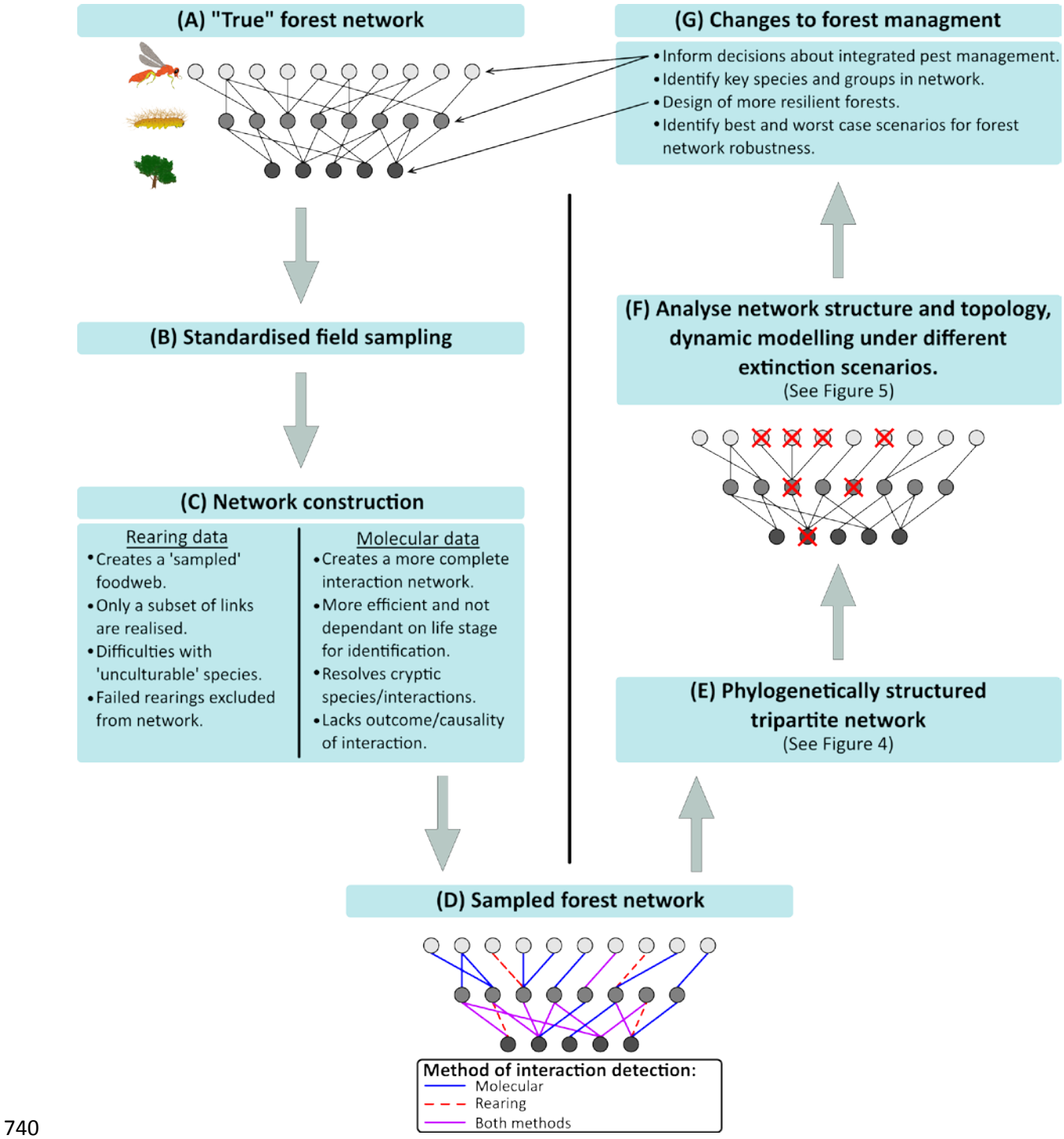

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741 Figure 1. The steps involved in constructing and analysing large, phylogenetically structured
742 species-interaction networks to inform forest management, here considering a plant-herbivore-
743 parasitoid network, but applicable to any ecological network. In order to create a complete,
744 tripartite network (A), forest plants and arthropods are sampled using standard census
745 techniques (B) and their interactions are determined through traditional identification and
746 rearing, and/or molecular approaches (C), both of which have advantages and disadvantages,
747 but which when combined result in the closest approximation to the 'true' forest network (D).
748 Interactions can be determined using both approaches, but many more (particularly difficult to
749 observe interactions) can be detected using nested tagging metabarcoding and the information
750 generated used to create phylogenetically structured networks (E). The structure and topology
751 of the network can then be analysed and computer modelling used to determine the robustness
752 of the networks to simulated species extinctions (F). Network analysis can be used to inform
753 current forest management, such as targeted pest management, determine the ecological
754 consequences of species loss as well as to suggest a tree species composition that will
755 maximise the robustness of future forests (G).
756

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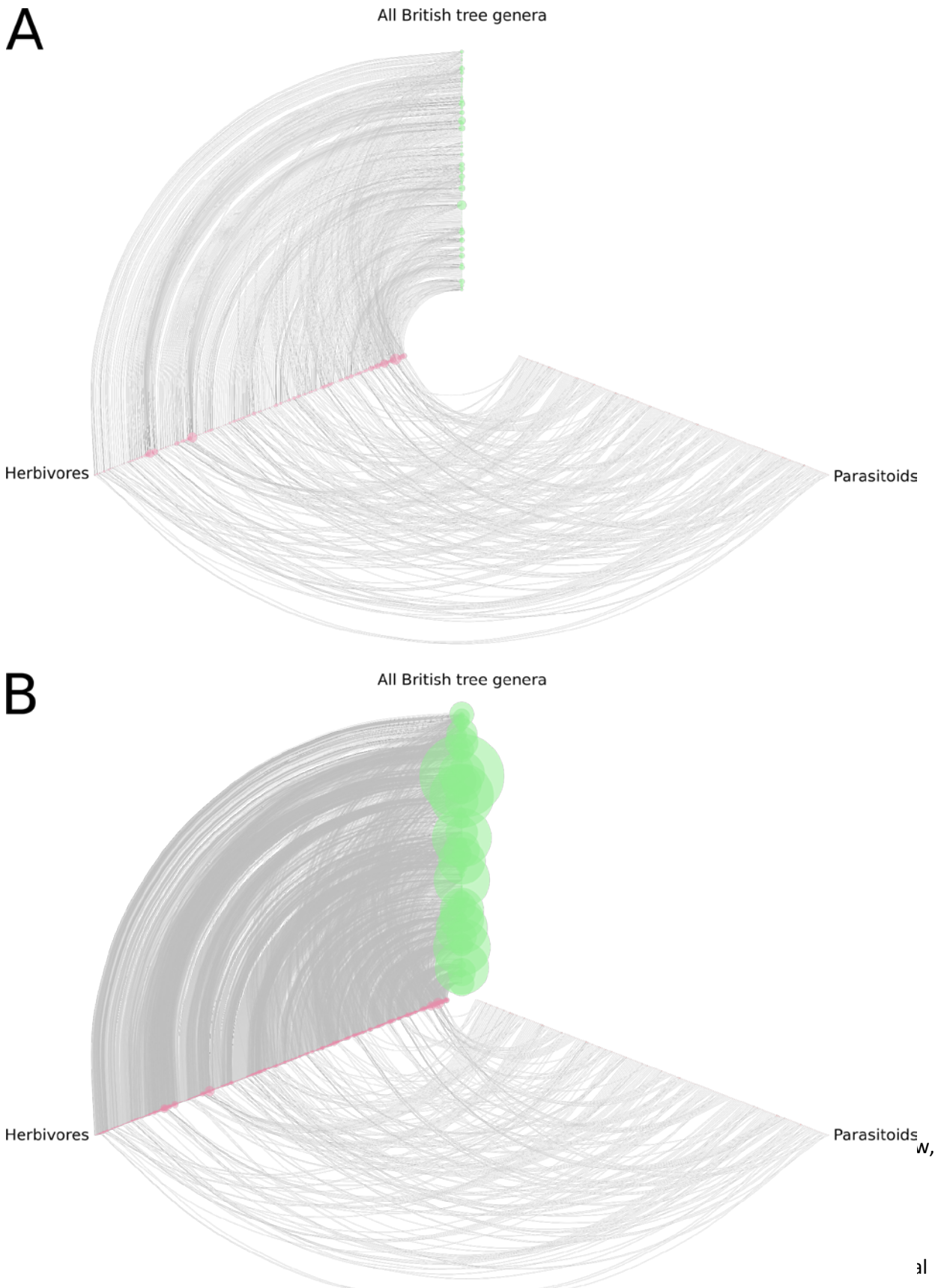

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758 Figure 2. Tritrophic hive plots of native British tree genera, their herbivores and parasitoids. (A)
759 contains only those herbivore species for which parasitoid interactions have been recorded,
760 while (B) contains all known plant-herbivore interactions. Node sizes are scaled by the number
761 of links connecting to them. An explanation of how this diagram has been created is available in
762 the supplementary information.
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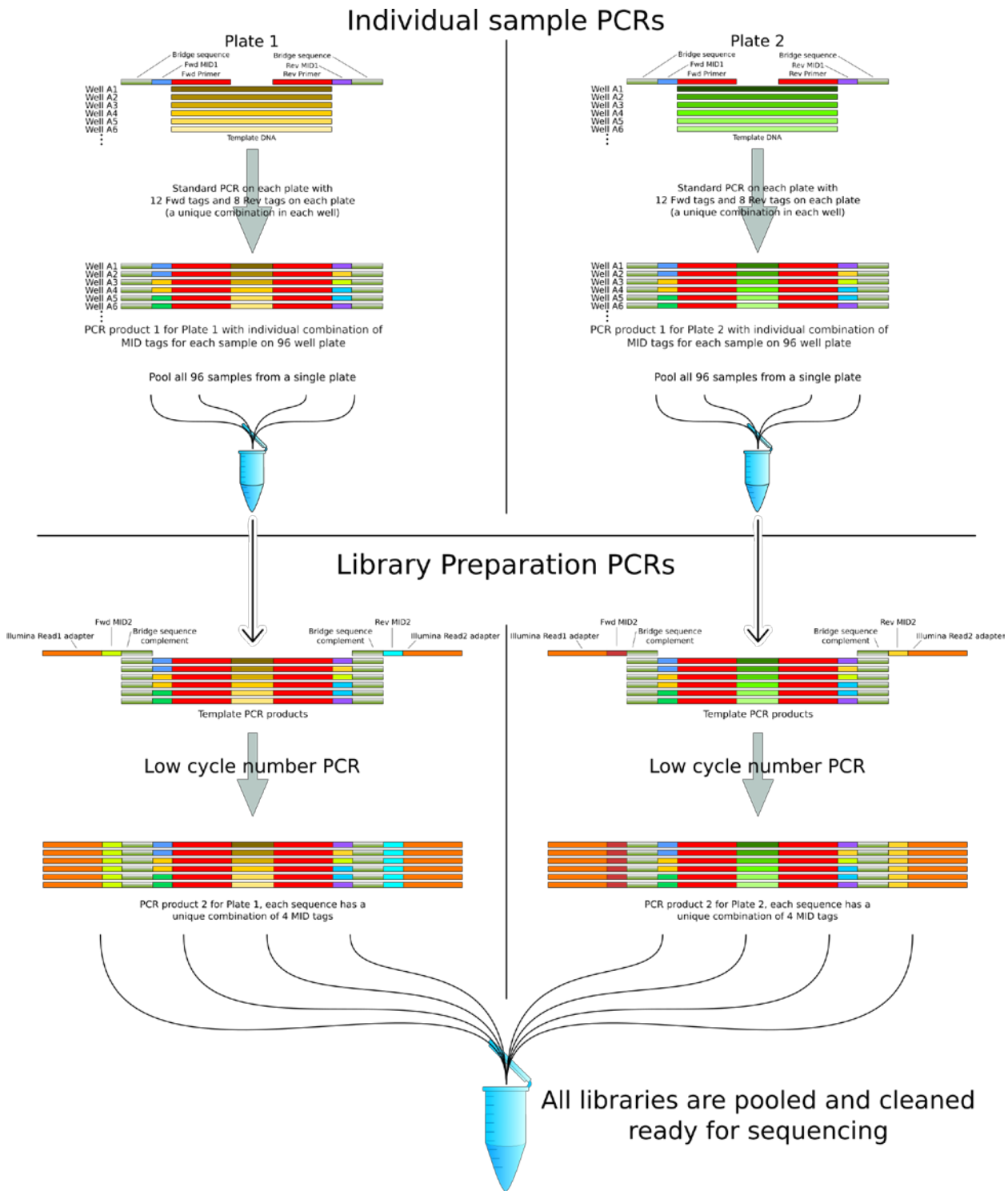

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765 Figure 3. The tagging and pooling regime required for 'nested tagging' Illumina barcoding.
766 Universal primers with MID tags are used to selectively amplify part of the COX1 barcode region
767 and individually tag each individual on a plate. A PCR based library preparation protocol is then
768 used to both add MID tags for each plate and add the Illumina plate adapters for sequencing.
769 This approach has recently been used to construct host-parasitoid networks on British oak trees
770 (Kitson *et al.* 2016- Submitted).
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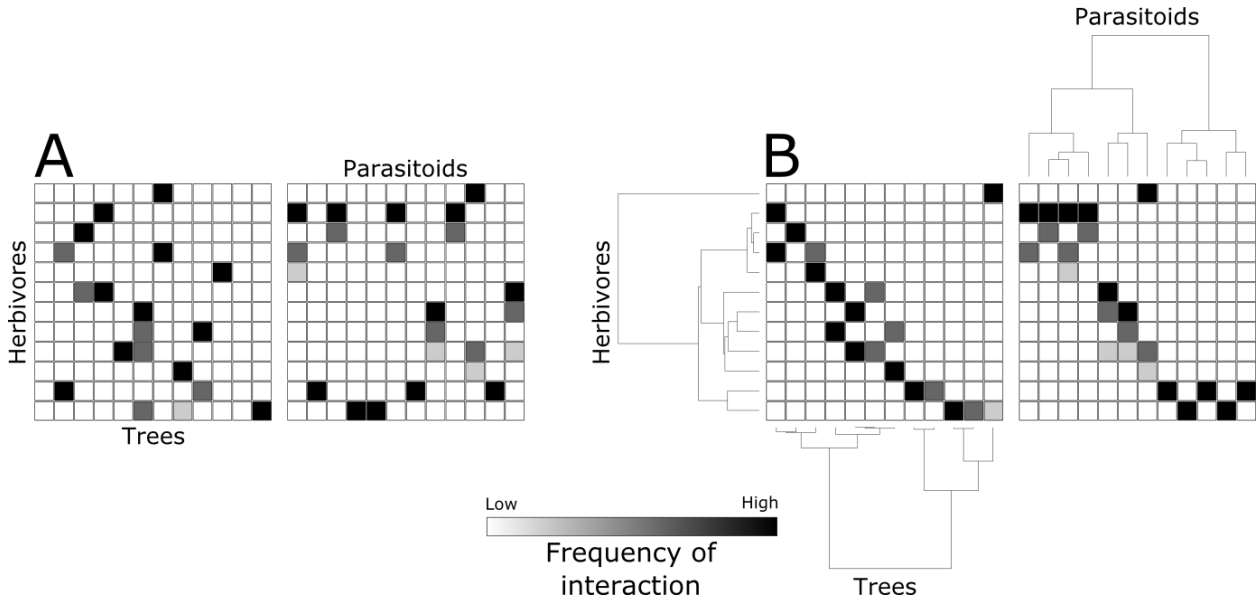

772
773 Figure 4. 'Nested tagging' metabarcoding provides additional data allowing ecological networks
774 to be phylogenetically structured. For illustrative purposes, (A) shows the supposed tree-
775 herbivore and herbivore-parasitoid interactions based on traditional field observations and insect
776 rearing. The frequency of interaction between species is shown by shading, the darker the
777 shading the higher the frequency. By adding the hypothetical phylogenies of the trees,
778 herbivores and parasitoids to the matrices (B), it is possible to investigate the presence of

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779 phylogenetic signals in the ecological networks and variation within and between trophic levels
780 (see Elias *et al.* 2013 for an example across 4 trophic levels). Such information can be used to
781 determine extinction scenarios in robustness analyses.
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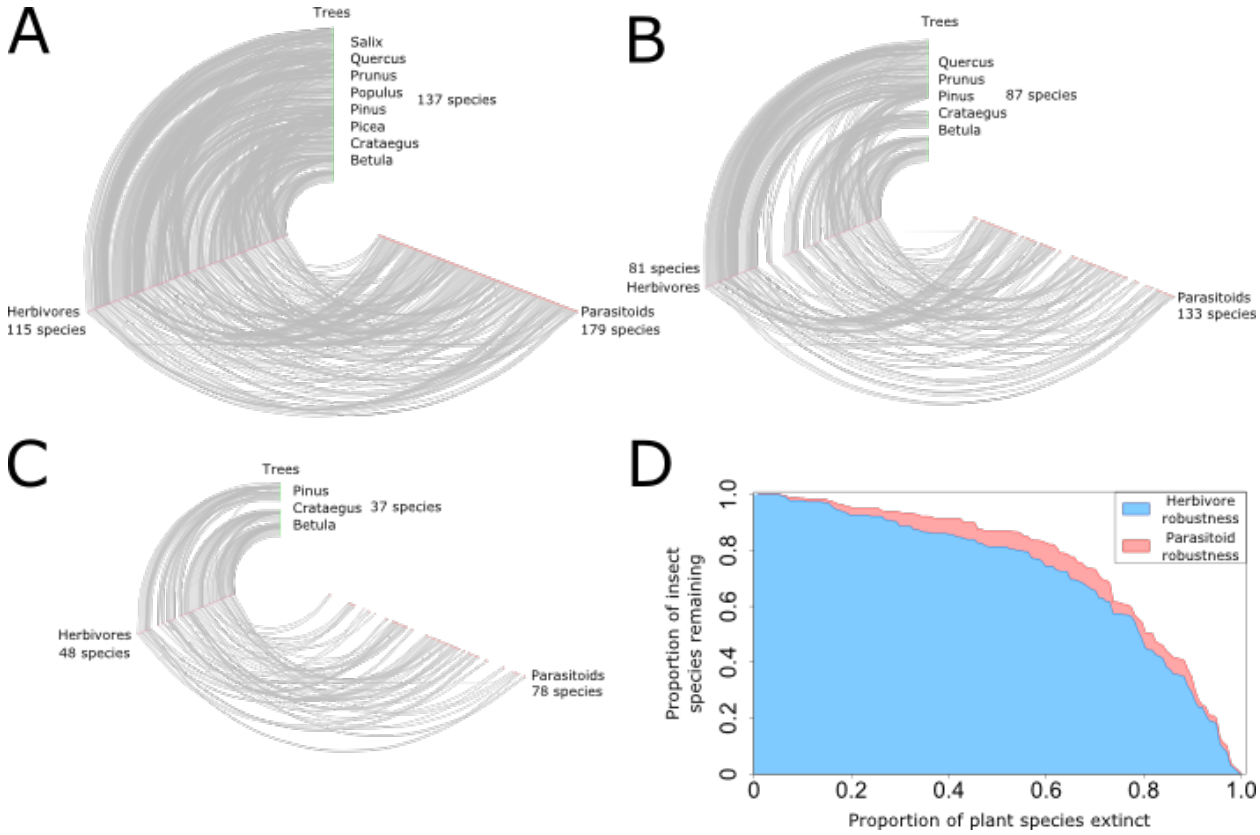

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784 Figure 5. Tree loss has consequences across trophic levels. Tree genera have been selected to
785 include the 8 most frequently featured in the DBIF database showing: (A) all interactions
786 between the selected tree genera and their herbivores with known parasitoids; (B) and (C)
787 successive random tree extinction; and (D) the cascading extinctions across trophic levels. An
788 explanation of how this diagram has been created is available in the supplementary information.

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