



## Sampling networks of ecological interactions

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# Sampling networks of ecological interactions

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## Summary

1. Sampling ecological interactions presents similar challenges, problems, potential biases, and constraints as sampling individuals and species in biodiversity inventories. Robust estimates of the actual number of interactions (links) within diversified ecological networks require adequate sampling effort that needs to be explicitly gauged. Yet we still lack a sampling theory explicitly focusing on ecological interactions.
2. While the complete inventory of interactions is likely impossible, a robust characterization of its main patterns and metrics is probably realistic. We

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must acknowledge that a sizeable fraction of the maximum number of interactions  $I_{max}$  among, say,  $A$  animal species and  $P$  plant species (i.e.,  $I_{max} = AP$ ) is impossible to record due to forbidden links, i.e., life-history restrictions. Thus, the number of observed interactions  $I$  in robustly sampled networks is typically  $I \ll I_{max}$ , resulting in sparse interaction matrices with low connectance.

3. Here I provide a review and outline a conceptual framework for interaction sampling by building an explicit analogue to individuals and species sampling, thus extending diversity-monitoring approaches to the characterization of complex networks of ecological interactions. Contrary to species inventories, a sizable fraction of non-observed pairwise interactions cannot be sampled, due to biological constraints that forbid their occurrence.
4. Reasons for forbidden links are multiple but mainly stem from spatial and temporal uncoupling, size mismatches, and intrinsically low probabilities of interspecific encounter for most potential interactions of partner species. Adequately assessing the completeness of a network of ecological interactions thus needs knowledge of the natural history details embedded, so that forbidden links can be accounted for as a portion of the unobserved links when addressing sampling effort.
5. Recent implementations of inference methods for unobserved species or for individual-based data can be combined with the assessment of forbidden links. This can help in estimating their relative importance, simply by the difference between the asymptotic estimate of interaction richness *in a robustly-sampled* assemblage and the maximum richness  $I_{max}$  of interactions.

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This is crucial to assess the rapid and devastating effects of defaunation-driven loss of key ecological interactions and the services they provide and the analogous losses related to interaction gains due to invasive species and biotic homogenization.

Keywords

complex networks, food webs, frugivory, mutualism, plant-animal interactions, pollination, seed dispersal

Introduction

Biodiversity sampling is a labour-intensive activity, and sampling is often not sufficient to detect all or even most of the species present in an assemblage. Gotelli & Colwell (2011).

1 Biodiversity species assessment aims at sampling individuals in collections and  
2 determining the number of species represented. Given that, by definition, samples  
3 are incomplete, these collections do not enumerate the species actually present.  
4 The ecological literature dealing with robust estimators of species richness and di-  
5 versity in collections of individuals is immense, and a number of useful approaches  
6 have been used to obtain such estimates (Magurran, 1988; Gotelli & Colwell, 2001;  
7 Colwell *et al.*, 2004; Hortal *et al.*, 2006; Colwell, 2009; Gotelli & Colwell, 2011; Chao  
8 *et al.*, 2014). Recent effort has been also focused at defining essential biodiversity  
9 variables (EBV) (Pereira *et al.*, 2013) that can be sampled and measured repeat-

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10 edly to complement biodiversity estimates. Yet sampling species or taxa-specific  
11 EBVs is just probing a single component of biodiversity; interactions among species  
12 are another fundamental component, one that supports the existence, but in some  
13 cases also the extinction, of species. For example, the extinction of interactions  
14 represents a dramatic loss of biodiversity because it entails the loss of fundamen-  
15 tal ecological functions (Valiente-Banuet *et al.*, 2014). This missed component of  
16 biodiversity loss, the extinction of ecological interactions, very often accompanies,  
17 or even precedes, species disappearance. Interactions among species are thus a key  
18 component of biodiversity and here I aim to show that most problems associated  
19 with sampling interactions in natural communities relate to, and are even worse  
20 than, problems associated with sampling species diversity. I consider pairwise in-  
21 teractions among species at the habitat level, in the context of alpha diversity and  
22 the estimation of local interaction richness from sampling data (Chao *et al.*, 2014).  
23 In the first part I provide a succinct overview of previous work addressing sampling  
24 issues for ecological interaction networks. In the second part, I discuss specific ra-  
25 tionales for sampling the biodiversity of ecological interactions. Finally, I provide  
26 a short overview of asymptotic diversity estimates (Gotelli & Colwell, 2001), and  
27 a discussion of its application to interaction sampling. Most of the examples come  
28 from the analysis of plant-animal interaction networks, yet are applicable to other  
29 types of interspecific interactions.

30 Interactions can be a much better indicator of the richness and diversity of  
31 ecosystem functions than a simple list of taxa and their abundances and/or related  
32 biodiversity indicator variables (EBVs) (Memmott *et al.*, 2006; Valiente-Banuet  
33 *et al.*, 2014). Thus, sampling interactions should be a central issue when identi-  
34 fying and diagnosing ecosystem services (e.g., pollination, seeding by frugivores,

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etc.). Fortunately, the whole battery of biodiversity-related tools used by ecologists to sample biodiversity (species, *sensu stricto*) can be extended and applied to the sampling of interactions (see Table 2 in Colwell *et al.*, 2004). Monitoring interactions is a type of biodiversity sampling and is subject to similar methodological shortcomings, especially under-sampling (Jordano, 1987; Jordano *et al.*, 2009; Vázquez *et al.*, 2009; Dorado *et al.*, 2011; Rivera-Hutinel *et al.*, 2012). We are interested in having a complete list of all the pairwise interactions among species (e.g., all the distinct, species-species interactions, or links, among the pollinators and flowering plants) that do actually exist in a given community. Sampling these interactions thus entails exactly the same problems, limitations, constraints, and potential biases as sampling individual organisms and species diversity. As Mao & Colwell (2005) put it, these are the workings of Preston’s demon, the moving “veil line” (Preston, 1948) between the detected and the undetected interactions as sample size increases.

Early efforts to recognize and solve sampling problems in analyses of interactions stem from research on food webs and to determine how undersampling biases food web metrics (Martinez, 1991; Cohen *et al.*, 1993; Wells & O’Hara, 2012, among others). In addition, the myriad of classic natural history studies documenting animal diets, host-pathogen infection records, plant herbivory records, etc., represent efforts to document interactions occurring in nature. All of them share the problem of sampling incompleteness influencing the patterns and metrics reported. Yet, despite the early recognition that incomplete sampling may seriously bias the analysis of ecological networks (Jordano, 1987), only recent studies have explicitly acknowledged it and attempted to determine its influence (Ollerton & Cranmer, 2002; Nielsen & Bascompte, 2007; Vázquez *et al.*, 2009; Gibson *et al.*,

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2011; Olesen *et al.*, 2011; Chacoff *et al.*, 2012; Rivera-Hutinel *et al.*, 2012; Olito & Fox, 2014; Bascompte & Jordano, 2014; Vizentin-Bugoni *et al.*, 2014, 2016; Frund *et al.*, 2016). The sampling approaches have been extended to predict patterns of coextinctions in interaction assemblages (e.g., hosts-parasites) (Colwell *et al.*, 2012). Most empirical studies provide no indication of sampling effort, implicitly assuming that the reported network patterns and metrics are robust. Yet recent evidences point out that number of partner species detected, number of actual links, and some aggregate statistics describing network patterns, are prone to sampling bias (Nielsen & Bascompte, 2007; Dorado *et al.*, 2011; Olesen *et al.*, 2011; Chacoff *et al.*, 2012; Rivera-Hutinel *et al.*, 2012; Olito & Fox, 2014; Frund *et al.*, 2016). Most of these evidences, however, come either from simulation studies (Frund *et al.*, 2016) or from relatively species-poor assemblages. Most certainly, sampling limitations pervade biodiversity inventories and we might rightly expect that frequent interactions may be over-represented and rare interactions may be missed entirely in studies of mega-diverse assemblages (Bascompte & Jordano, 2014); but, to what extent?

## Sampling interactions: methods

When we sample interactions in the field we record the presence of two species that interact in some way. For example, Snow and Snow(1988) recorded an interaction whenever they saw a bird “touching” a fruit on a plant. We observe and record feeding observations, visitation, occupancy, presence in pollen loads or in fecal samples, etc., of *individual* animals or plants and accumulate pairwise interactions, i.e., lists of species partners and the frequencies with which we observe them. We

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83 assume that the matrix (species numbers) is predefined (i.e., all species interacting  
84 are well documented).

85 Most types of ecological interactions can be illustrated with bipartite graphs,  
86 with two or more distinct groups of interacting partners (Bascompte & Jordano,  
87 2014); for illustration purposes I'll focus more specifically on plant-animal inter-  
88 actions. Sampling interactions requires filling the cells of an interaction matrix  
89 with data. The matrix,  $\Delta = AP$  (the adjacency matrix for the graph representa-  
90 tion of the network), is a 2D inventory of the interactions among, say,  $A$  animal  
91 species (rows) and  $P$  plant species (columns) (Jordano, 1987; Bascompte & Jor-  
92 dano, 2014). The matrix entries illustrate the values of the pairwise interactions  
93 visualized in the  $\Delta$  matrix, and can be 0 or 1, for presence-absence of a given  
94 pairwise interaction, or take a quantitative weight  $w_{ji}$  to represent the interaction  
95 intensity or unidirectional effect of species  $j$  on species  $i$  (Bascompte & Jordano,  
96 2014; Vázquez *et al.*, 2015). The outcomes of most ecological interactions are  
97 dependent on frequency of encounters (e.g., visit rate of pollinators, number of  
98 records of ant defenders, frequency of seeds in fecal samples). Thus, a frequently  
99 used proxy for interaction intensities  $w_{ji}$  is just how frequent new interspecific  
100 encounters are, whether or not appropriately weighted to estimate interaction ef-  
101 fectiveness (Vázquez *et al.*, 2005).

102 We need to define two basic steps in the sampling of interactions: 1) which  
103 type of interactions we sample; and 2) which type of record we get to document  
104 the existence of an interaction. In step #1 we need to take into account whether  
105 we are sampling the whole community of interactor species (all the animal and  
106 plant species) or just a subset of them, i.e., a sub matrix  $\Delta_{m,n}$  of  $m < A$  animal  
107 species and  $n < P$  plant species of the adjacency matrix  $\Delta_{AP}$ . Subsets can be:



108 a) all the potential plants interacting with a subset of the animals (Fig. 1a); b)  
109 all the potential animal species interacting with a subset of the plant species (Fig.  
110 1b); c) a subset of all the potential animal species interacting with a subset of all  
111 the plant species (Fig. 1c). While some discussion has considered how to establish  
112 the limits of what represents a network (Strogatz, 2001) (in analogy to discussion  
113 on food-web limits; Cohen, 1978), it must be noted that situations a-c in Fig. 1  
114 do not represent complete interaction networks. Subnet sampling is generalized  
115 in studies of biological networks (e.g., protein interactions, gene regulation), yet  
116 it is important to recognize that most properties of subnetworks (even random  
117 subsamples) do not represent properties of whole networks (Stumpf *et al.*, 2005).

118 In step #2 above we face the problem of the type of record we take to sample  
119 interactions. This is important because it defines whether we approach the problem  
120 of filling up the interaction matrix in a “zoo-centric” way or in a “phyto-centric”  
121 way. Zoo-centric studies directly sample animal activity and document the plants  
122 ‘touched’ by the animal. For example, analysis of pollen samples recovered from the  
123 body of pollinators, analysis of fecal samples of frugivores, radio-tracking data, etc.  
124 Phyto-centric studies take samples of focal individual plant species and document  
125 which animals ‘arrive’ or ‘touch’ the plants. Examples include focal watches of  
126 fruiting or flowering plants to record visitation by animals, raising insect herbivores  
127 from seed samples, identifying herbivory marks in samples of leaves, etc.

128 Most recent analyses of plant-animal interaction networks are phyto-centric;  
129 just 3.5% of available plant-pollinator ( $N=58$ ) or 36.6% plant-frugivore ( $N=22$ )  
130 interaction datasets are zoo-centric (see Schleuning *et al.*, 2012). Moreover, most  
131 available datasets on host-parasite (parasitoid) or plant-herbivore interactions are  
132 “host-centric” or phyto-centric (e.g., Thébaud & Fontaine, 2010; Morris *et al.*,

2013; Eklöf *et al.*, 2013). This may be related to a variety of causes, like preferred methodologies by researchers working with a particular group or system, logistic limitations, or inherent taxonomic focus of the research questions. A likely result of phyto-centric sampling would be adjacency matrices with large  $A : P$  ratios. In contrast, zoo-centric samplings might be prone to detect plants from outside the habitat, complicating the definition of network boundaries. In any case we don't have a clear view of the potential biases that taxa-focused sampling may generate in observed network patterns, for example by generating consistently asymmetric interaction matrices (Dormann *et al.*, 2009).

Reasonably complete analyses of interaction networks can be obtained when combining both phyto-centric and zoo-centric sampling. For example, Bosch *et al.* (2009) showed that the addition of pollen load data on top of focal-plant sampling of pollinators unveiled a significant number of interactions, resulting in important network structural changes. Olesen *et al.* (2011) identified pollen loads on sampled insects and added the new links to an observation-based visitation matrix, with an extra 5% of links representing the estimated number of missing links in the pollination network. The overlap between observational and pollen-load recorded links was only 33%, underscoring the value of combining methodological approaches. Zoo-centric sampling has recently been extended with the use of DNA-barcoding, for example with plant-herbivore (Jurado-Rivera *et al.*, 2009), host-parasitoid (Wirta *et al.*, 2014; Evans *et al.*, 2016), and plant-frugivore interactions (González-Varo *et al.*, 2014). For mutualistic networks we would expect that zoo-centric sampling could help unveiling interactions of the animals with rare plant species or for relatively common plants species which are difficult to sample by direct observation. Future methodological work may provide signifi-

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cant advances showing how mixing different sampling strategies strengthens the completeness of network data. These mixed strategies may combine, for instance, timed watches at focal plants, spot censuses along walked transects, pollen load or seed contents analyses, monitoring with camera traps, and DNA barcoding records. However, there are no tested protocols and/or sampling designs for ecological interaction studies to suggest an optimum combination of approaches. Ideally, pilot studies would provide adequate information for each specific study setting.

## Sampling interactions: rationale

The number of distinct pairwise interactions that we can record in a landscape (an area of relatively homogeneous vegetation) is equivalent to the number of distinct classes in which we can classify the recorded encounters among *individuals* of two different species. Yet, individual-based interaction networks have been only recently studied (Dupont *et al.*, 2011; Wells & O'Hara, 2012). The most usual approach has been to pool individual-based interaction data into species-based summaries, an approach that ignores the fact that only a fraction of individuals may actually interact given a per capita interaction effect (Wells & O'Hara, 2012). Wells & O'Hara (2012) illustrate the pros and cons of the approach. We walk in the forest and see a blackbird  $Tm$  picking an ivy  $Hh$  fruit and ingesting it: we have a record for  $Tm - Hh$  interaction. We keep advancing and record again a blackbird feeding on hawthorn  $Cm$  fruits so we record a  $Tm - Cm$  interaction; as we advance we encounter another ivy plant and record a blackcap swallowing a fruit so we now have a new  $Sa - Hh$  interaction, and so on. At the end we have a series of classes (e.g.,  $Sa - Hh$ ,  $Tm - Hh$ ,  $Tm - Cm$ , etc.), along with their

181 observed frequencies.

182 We get a vector  $c = [c_1 \dots c_n]'$  where  $c_j$  is the number of classes represented  $j$   
 183 times in our sampling:  $c_1$  is the number of singletons (interactions recorded once),  
 184  $c_2$  is the number of twin pairs (interactions with just two records),  $c_3$  the number  
 185 of triplets, etc. The problem thus turns to be estimating the number of distinct  
 186 classes  $C$  from the vector of  $c_j$  values and the frequency of unobserved interactions  
 187 (see “The real missing links” below).

188 More specifically, we usually obtain a type of reference sample (Chao *et al.*,  
 189 2014) for interactions: a series of repeated samples (e.g., observation days, 1h  
 190 watches, etc.) with quantitative information, i.e., recording the number of in-  
 191 stances of each interaction type on each day. This replicated abundance data,  
 192 can be treated in three ways: 1) Abundance data within replicates: the counts  
 193 of interactions, separately for each day; 2) Pooled abundance data: the counts of  
 194 interactions, summed over all days (the most usual approach); and 3) Replicated  
 195 incidence data: the number of days on which we recorded each interaction. As-  
 196 suming a reasonable number of replicates, replicated incidence data is considered  
 197 to be the most robust statistically, as it takes account of heterogeneity among days  
 198 (Colwell *et al.*, 2004, 2012; Chao *et al.*, 2014). Thus, both presence-absence and  
 199 weighted information on interactions can be accommodated for this purpose.

## 200 The species assemblage

201 When we consider an observed and recorded sample of interactions on a particular  
 202 assemblage of  $A_{obs}$  and  $P_{obs}$  species (or a set of replicated samples) as a reference  
 203 sample (Chao *et al.*, 2014) we may have three sources of undersampling error.

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204 These sources are ignored if we treat the reference sample as a true representation  
 205 of the interactions in a well-defined assemblage: 1) some animal species are actually  
 206 present but not observed (zero abundance or incidence in the interactions in the  
 207 reference sample),  $A_0$ ; 2) some plant species are actually present but not observed  
 208 (zero abundance or incidence in the interactions in the reference sample),  $P_0$ ; 3)  
 209 some unobserved links (the zeroes in the adjacency matrix,  $UL$ ) may actually  
 210 occur but not recorded. Thus a first problem is determining if  $A_{obs}$  and  $P_{obs}$  truly  
 211 represent the actual species richness interacting in the assemblage. To this end  
 212 we might use the replicated reference samples to estimate the true number of  
 213 interacting animal  $A_{est}$  and plant  $P_{est}$  species as in traditional diversity estimation  
 214 analysis (Chao *et al.*, 2014). If there are no uniques (species seen on only one day),  
 215 then  $A_0$  and  $P_0$  will be zero (based on the Chao2 formula), and we have  $A_{obs}$  and  
 216  $P_{obs}$  as robust estimates of the actual species richness of the assemblage. If  $A_0$   
 217 and  $P_0$  are not zero they estimate the minimum number of undetected animal and  
 218 plant species that can be expected with a sufficiently large number of replicates,  
 219 taken from the same assemblage/locality by the same methods in the same time  
 220 period. We can use extrapolation methods (Colwell *et al.*, 2012) to estimate how  
 221 many additional replicate surveys it would take to reach a specified proportion  $g$   
 222 of  $A_{est}$  and  $P_{est}$ .

## 223 The interactions

224 We are then faced with assessing the sampling of interactions  $I$ . Table 1 summa-  
 225 rizes the main components and targets for estimation of interaction richness. In  
 226 contrast with traditional species diversity estimates, sampling networks has the

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paradox that despite the potentially interacting species being present in the sampled assemblage (i.e., included in the  $A_{obs}$  and  $P_{obs}$  species lists), some of their pairwise interactions are impossible to record. The reason is forbidden links. Independently of whether we sample full communities or subset communities we face a problem: some of the interactions that we can visualize in the empty adjacency matrix  $\Delta$  will simply not occur. With a total of  $A_{obs}P_{obs}$  “potential” interactions (eventually augmented to  $A_{est}P_{est}$  in case we have undetected species), a fraction of them are impossible to record, because they are forbidden (Jordano *et al.*, 2003; Olesen *et al.*, 2011).

Our goal is to estimate the true number of non-null  $AP$  interactions, including interactions that actually occur but have not been observed ( $I_0$ ) from the replicated incidence frequencies of interaction types:  $I_{est} = I_{obs} + I_0$ . Note that  $I_0$  estimates the minimum number of undetected plant-animal interactions that can be expected with a sufficiently large number of replicates, taken from the same assemblage/locality by the same methods in the same time period. Therefore we have two types of non-observed links:  $UL^*$  and  $UL$ , corresponding to the real assemblage species richness and to the observed assemblage species richness, respectively (Table 1).

Forbidden links are non-occurrences of pairwise interactions that can be accounted for by biological constraints, such as spatio-temporal uncoupling (Jordano, 1987), size or reward mismatching, foraging constraints (e.g., accessibility) (Moré *et al.*, 2012), and physiological-biochemical constraints (Jordano, 1987). We still have very little information about the frequency of forbidden links in natural communities (Jordano *et al.*, 2003; Stang *et al.*, 2009; Vázquez *et al.*, 2009; Olesen *et al.*, 2011; Ibanez, 2012; Maruyama *et al.*, 2014; Vizentin-Bugoni *et al.*, 2014)

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(Table 1). Forbidden links are thus represented as structural zeroes in the interaction matrix, i.e., matrix cells that cannot get a non-zero value. Therefore, we need to account for the frequency of these structural zeros in our matrix before proceeding.

Our main problem then turns to estimate the number of true missed links, i.e., those that can't be accounted for by biological constraints and that might suggest undersampling. Thus, the sampling of interactions in nature, as the sampling of species, is a cumulative process. In our analysis, we are not re-sampling individuals, but interactions, so we built interaction-based accumulation curves. We add new, distinct, interactions recorded as we increase sampling effort (Fig. 2, and Supplementary Online Material). We can obtain an Interaction Accumulation Curve (*IAC*) analogous to a Species Curve (*SAC*) (see Supporting Information in the online data availability repository): the observed number of distinct pairwise interactions in a survey or collection as a function of the accumulated number of observations or samples (Colwell, 2009).

## Empirical data on Forbidden Links

Adjacency matrices are frequently sparse, i.e., they are densely populated with zeroes, with a fraction of them being structural (unobservable interactions) (Bascompte & Jordano, 2014). Thus, it would be a serious interpretation error to attribute the sparseness of adjacency matrices for bipartite networks to just the result of undersampling. The actual typology of link types in ecological interaction networks is thus more complex than just the two categories of observed and unobserved interactions (Table 1). Unobserved interactions are represented by

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275 zeroes and belong to two categories. Missing interactions may actually exist but  
 276 require additional sampling or a variety of methods to be observed. Forbidden  
 277 links, on the other hand, arise due to biological constraints limiting interactions  
 278 and remain unobservable in nature, irrespectively of sampling effort (Table 1).  
 279 Forbidden links *FL* may actually account for a relatively large fraction of unob-  
 280 served interactions *UL* when sampling taxonomically-restricted subnetworks (e.g.,  
 281 plant-hummingbird pollination networks) (Table 1). Phenological uncoupling is  
 282 also prevalent in most networks, and may add up to explain ca. 25-40% of the for-  
 283 bidden links, especially in highly seasonal habitats, and up to 20% when estimated  
 284 relative to the total number of unobserved interactions (Table 2). In any case, we  
 285 might expect that a fraction of the missing links *ML* would be eventually explained  
 286 by further biological reasons, depending on the knowledge of natural details of the  
 287 particular systems. Our goal as naturalists would be to reduce the fraction of *UL*  
 288 which remain as missing links; to this end we might search for additional biological  
 289 constraints or increase sampling effort. For instance, habitat use patterns by hum-  
 290 mingbirds in the Arima Valley network (Table 2; Snow & Snow, 1972) impose a  
 291 marked pattern of microhabitat mismatches causing up to 44.5% of the forbidden  
 292 links. A myriad of biological causes beyond those included as *FL* in Table 1 may  
 293 contribute explanations for *UL*: limits of color perception, presence of secondary  
 294 metabolites in fruit pulp and leaves, toxins and combinations of monosaccharides  
 295 in nectar, etc. For example, aside from *FL*, some pairwise interactions may sim-  
 296 ply have an asymptotically-zero probability of interspecific encounter between the  
 297 partner species, if they are very rare. However, it is surprising that just the limited  
 298 set of forbidden link types considered in Table 1 explain between 24.6-77.2% of  
 299 the unobserved links. Notably, the Arima Valley, Santa Virgínia, and Hato Ratón



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300 networks have  $> 60\%$  of the unobserved links explained, which might be related  
 301 to the fact that they are subnetworks (Arima Valley, Santa Virgínia) or relatively  
 302 small networks (Hato Ratón). All this means that empirical networks may have  
 303 sizable fractions of structural zeroes. Ignoring this biological fact may contribute  
 304 to wrongly inferring undersampling of interactions in real-world assemblages.

305 To sum up, two elements of inference are required in the analysis of unobserved  
 306 interactions in ecological interaction networks: first, detailed natural history infor-  
 307 mation on the participant species that allows the inference of biological constraints  
 308 imposing forbidden links, so that structural zeroes can be identified in the adja-  
 309 cency matrix. Second, a critical analysis of sampling robustness and a robust  
 310 estimate of the actual fraction of missing links,  $M$ , resulting in a robust estimate  
 311 of  $I$ . In the next sections we explore these elements of inference, using  $IAC$ s as  
 312 analogs to  $SAC$ s to assess the robustness of interaction sampling.

## 313 **Assessing sampling effort when recording interac-** 314 **tions: asymptotic diversity estimates**

315 A plot of the cumulative number of species recorded,  $S_n$ , as a function of some  
 316 measure of sampling effort (say,  $n$  samples taken) yields the species accumulation  
 317 curve (SAC) or collector's curve (Colwell & Coddington, 1994). Similarly, inter-  
 318 action accumulation curves (IAC), analogous to SACs (Gotelli & Colwell, 2001;  
 319 Hortal *et al.*, 2006; Chao, 2005; Colwell, 2013), can be used to assess the robust-  
 320 ness of interactions sampling for plant-animal community datasets (Jordano, 1987;  
 321 Jordano *et al.*, 2009; Olesen *et al.*, 2011; Chacoff *et al.*, 2012).

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322 The basic method to estimate sampling effort and explicitly show the analogues  
 323 with rarefaction analysis in biodiversity research is to vectorize the interaction ma-  
 324 trix  $AP$  so that we get a vector of all the potential pairwise interactions ( $I_{max}$ ,  
 325 Table 1) that can occur in the observed assemblage with  $A_{obs}$  animal species and  
 326  $P_{obs}$  plant species. The new “species” we aim to sample are the pairwise interactions  
 327 (Table 3), as previously discussed. In general, if we have  $A = 1...i$ , animal species  
 328 and  $P = 1...j$  plant species (assuming a complete list of species in the assemblage),  
 329 we’ll have a vector of “new” species to sample:  $A_1P_1, A_1P_2, ...A_2P_1, A_2P_2, ...A_iP_j$ .  
 330 We can represent the successive samples where we can potentially get records of  
 331 these interactions in a matrix with the vectorized interaction matrix and columns  
 332 representing the successive samples we take (Table 3). This is simply a vectorized  
 333 version of the interaction matrix  $\Delta$ . This is analogous to a biodiversity sam-  
 334 pling matrix with species as rows and sampling units (e.g., quadrats) as columns  
 335 (Jordano *et al.*, 2009). The package *EstimateS* (Colwell, 2013) includes a com-  
 336 plete set of functions for estimating the mean IAC and its unconditional standard  
 337 deviation from random permutations of the data, or subsampling without replace-  
 338 ment (Gotelli & Colwell, 2001); it further reports asymptotic estimators for the  
 339 expected number of distinct pairwise interactions included in a given reference  
 340 sample of interaction records (see also the `specaccum` function in library `vegan`  
 341 of the R Package)(R Development Core Team, 2010; Jordano *et al.*, 2009; Olesen  
 342 *et al.*, 2011). In particular, we may take advantage of replicated incidence data,  
 343 as it takes account of heterogeneity among samples (days, censuses, etc.; R.K Col-  
 344 well, pers. comm.) (see also Colwell *et al.*, 2004, 2012; Chao *et al.*, 2014). Future  
 345 theoretical work will be needed to formally assess the similarities and differences  
 346 between the species vs. interactions sampling approaches and developing biologi-

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347 cally meaningful null models of expected interaction richness with added sampling  
348 effort.

349 Diversity-accumulation analysis (Magurran, 1988; Hortal *et al.*, 2006) comes  
350 up immediately with this type of dataset. This procedure plots the accumulation  
351 curve for the expected number of distinct pairwise interactions recorded with in-  
352 creasing sampling effort (Jordano *et al.*, 2009; Olesen *et al.*, 2011). Asymptotic  
353 estimates of interaction richness and its associated standard errors and confidence  
354 intervals can thus be obtained (Hortal *et al.*, 2006) (see Table 4 and Supplemen-  
355 tary Online Material). The characteristic feature of interaction datasets is that,  
356 due to forbidden links, a number of pairwise interactions among the  $I_{max}$  number  
357 specified in the  $\Delta$  adjacency matrix cannot be recorded, irrespective of sampling  
358 effort.

359 We may expect undersampling specially in moderate to large sized networks  
360 with multiple modules (i.e., species subsets requiring different sampling strategies)  
361 (Jordano, 1987; Olesen *et al.*, 2011; Chacoff *et al.*, 2012); adequate sampling may  
362 be feasible when interaction subwebs are studied (Olesen *et al.*, 2011; Vizentin-  
363 Bugoni *et al.*, 2014), typically with more homogeneous subsets of species (e.g.,  
364 bumblebee-pollinated flowers).

365 Mixture models incorporating detectabilities have been proposed to effectively  
366 account for rare species (Mao & Colwell, 2005). In an analogous line, mixture  
367 models could be extended to samples of pairwise interactions, also with specific  
368 detectability values. These detection rate/odds could be variable among groups of  
369 interactions, depending on their specific detectability. For example, detectability of  
370 flower-pollinator interactions involving bumblebees could have a higher detectabil-  
371 ity than flower-pollinator pairwise interactions involving, say, nitidulid beetles.

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372 These more homogeneous groupings of pairwise interactions within a network de-  
 373 fine modules (Bascompte & Jordano, 2014), so we might expect that interactions  
 374 of a given module (e.g., plants and their hummingbird pollinators; Fig. 1a) may  
 375 share similar detectability values, in an analogous way to species groups receiving  
 376 homogeneous detectability values in mixture models (Mao & Colwell, 2005). In  
 377 its simplest form, this would result in a sample with multiple pairwise interactions  
 378 detected, in which the number of interaction events recorded for each distinct  
 379 interaction found in the sample is recorded (i.e., a column vector in Table 3, cor-  
 380 responding to, say, a sampling day). The number of interactions recorded for the  
 381  $i_{th}$  pairwise interaction (i.e.,  $A_i P_j$  in Table 3),  $Y_i$  could be treated as a Poisson  
 382 random variable with a mean parameter  $\lambda_i$ , its detection rate. Mixture models  
 383 (Mao & Colwell, 2005) include estimates for abundance-based data (their analogs  
 384 in interaction sampling would be weighted data), where  $Y_i$  is a Poisson random  
 385 variable with detection rate  $\lambda_i$ . This is combined with the incidence-based model,  
 386 where  $Y_i$  is a binomial random variable (their analogous in interaction sampling  
 387 would be presence/absence records of interactions) with detection odds  $\lambda_i$ . Let  
 388  $T$  be the number of samples in an incidence-based data set. A Poisson/binomial  
 389 density can be written as (Mao & Colwell, 2005):

$$g(y; \lambda) = \begin{cases} \frac{\lambda^y}{y! e^\lambda} & [1] \\ \binom{T}{y} \frac{\lambda^y}{(1+\lambda)^T} & [2] \end{cases}$$

390 where [1] corresponds to a weighted network, and [2] to a qualitative network.

391 The detection rates  $\lambda_i$  depend on the relative abundances  $\phi_i$  of the interactions,  
 392 the probability of a pairwise interaction being detected when it is present, and the

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sample size (the number of interactions recorded), which, in turn, is a function of the sampling effort. Unfortunately, no specific sampling model has been developed along these lines for species interactions and their characteristic features. For example, a complication factor might be that interaction abundances,  $\phi_i$ , in real assemblages are a function of the abundances of interacting species that determine interspecific encounter rates; yet they also depend on biological factors that ultimately determine if the interaction occurs when the partner species are present. For example,  $\lambda_i$  should be set to zero for all  $FL$ . In its simplest form,  $\phi_i$  could be estimated from just the product of partner species abundances, an approach recently used as a null model to assess the role of biological constraints in generating forbidden links and explaining interaction patterns (Vizentin-Bugoni *et al.*, 2014). Yet more complex models (e.g., Wells & O'hara 2012; 2016) should incorporate not only interspecific encounter probabilities, but also interaction detectabilities, phenotypic matching and incidence of forbidden links. Mixture models are certainly complex and for most situations of evaluating sampling effort better alternatives include the simpler incidence-based rarefaction and extrapolation (Colwell *et al.*, 2012; Chao *et al.*, 2014).

## The *real* missing links

Given that a fraction of unobserved interactions can be accounted for by forbidden links, what about the remaining missing interactions? We have already discussed that some of these could still be related to unaccounted constraints, and still others would be certainly attributable to insufficient sampling. Would this always be the case? A crucial ecological aspect limiting interactions within multispecific assem-

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416 blages of distinct taxonomic relatedness (Fig. 2) is the probability of interspecific  
417 encounter, i.e., the probability that two individuals of the partner species actually  
418 encounter each other in nature.

419 Given log-normally distributed abundances of the two species groups, the ex-  
420 pected probabilities of interspecific encounter ( $PIE$ ) would be simply the product  
421 of the two lognormal distributions. Thus, we might expect that for very low  $PIE$   
422 values, pairwise interactions would be either extremely difficult to sample, or sim-  
423 ply do not occur in nature. Consider the Nava de las Correhuelas interaction  
424 web (NCH, Table 2, 4), with  $A = 36$ ,  $P = 25$ ,  $I = 181$ , and almost half of the  
425 unobserved interactions not accounted for by forbidden links, thus  $M = 53.1\%$   
426 (Jordano *et al.*, 2009). A sizable fraction of these possible but missing links would  
427 be simply not occurring in nature, most likely due to extremely low  $PIE$ , in fact  
428 asymptotically zero. Given the vectorized list of pairwise interactions for NCH, I  
429 computed the  $PIE$  values for each one by multiplying element-wise the two species  
430 abundance distributions. The  $PIE_{max} = 0.0597$ , being a neutral estimate, based  
431 on the assumption that interactions occur in proportion to the species-specific local  
432 abundances. With  $PIE_{median} < 1.4 \cdot 10^{-4}$  we may safely expect (note the quantile  
433 estimate  $Q_{75\%} = 3.27 \cdot 10^{-4}$ ) that a sizable fraction of these missing interactions  
434 may not occur according to this neutral expectation (Jordano, 1987; Olesen *et al.*,  
435 2011) (neutral forbidden links, *sensu* Canard *et al.*, 2012).

436 When we consider the vectorized interaction matrix, enumerating all pairwise  
437 interactions for the  $AP$  combinations, the expected probabilities of finding a given  
438 interaction can be estimated with a Good-Turing approximation (Good, 1953).  
439 The technique, developed by Alan Turing and I.J. Good with applications to lin-  
440 guistics and word analysis (Gale & Sampson, 1995) has been recently extended in

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441 novel ways for ecological analyses (Chao *et al.*, 2015). In our present context it  
 442 estimates the probability of recording an interaction of a hitherto unseen pair of  
 443 partners, given a set of past records of interactions between other species pairs.  
 444 Let a sample of  $N$  interactions so that  $n_r$  distinct pairwise interactions have ex-  
 445 actly  $r$  records. All Good-Turing estimators obtain the underlying frequencies of  
 446 events as:

$$P(X) = \frac{(N_X + 1)}{T} \left(1 - \frac{E(1)}{T}\right) \quad (1)$$

447 where  $X$  is the pairwise interaction,  $N_X$  is the number of times interaction  $X$   
 448 is recorded,  $T$  is the sample size (number of distinct interactions recorded) and  
 449  $E(1)$  is an estimate of how many different interactions were recorded exactly once.  
 450 Strictly speaking Equation (1) gives the probability that the next interaction type  
 451 recorded will be  $X$ , after sampling a given assemblage of interacting species. In  
 452 other words, we scale down the maximum-likelihood estimator  $\frac{n}{T}$  by a factor of  
 453  $\frac{1-E(1)}{T}$ . This reduces all the probabilities for interactions we have recorded, and  
 454 makes room for interactions we haven't seen. If we sum over the interactions we  
 455 have seen, then the sum of  $P(X)$  is  $1 - \frac{1-E(1)}{T}$ . Because probabilities sum to one,  
 456 we have the left-over probability of  $P_{new} = \frac{E(1)}{T}$  of seeing something new, where  
 457 new means that we sample a new pairwise interaction.

## 458 Discussion

459 Recent work has inferred that most data available for interaction networks are  
 460 incomplete due to undersampling, resulting in a variety of biased parameters and

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461 network patterns (Chacoff *et al.*, 2012). It is important to note, however, that in  
 462 practice, most surveyed networks to date have been subnets of much larger net-  
 463 works. This is also true for protein interaction, gene regulation, and metabolic  
 464 networks, where only a subset of the molecular entities in a cell have been sam-  
 465 pled (Stumpf *et al.*, 2005). Despite recent attempts to document whole ecosystem  
 466 meta-networks (Pocock *et al.*, 2012), it is likely that most ecological interaction  
 467 networks will illustrate just major ecosystem compartments. Due to their high  
 468 generalization, high temporal and spatial turnover, and high complexity of associ-  
 469 ation patterns, adequate sampling of ecological interaction networks is challenging  
 470 and requires extremely large sampling effort. Undersampling of ecological net-  
 471 works may originate from the analysis of assemblage subsets (e.g., taxonomically  
 472 or functionally defined), and/or from logistically-limited sampling effort. It is ex-  
 473 tremely hard to robustly sample the set of biotic interactions even for relatively  
 474 simple, species-poor assemblages; thus, we need to assess how robust is the char-  
 475 acterization of the adjacency matrix  $\Delta$ . Concluding that an ecological network  
 476 dataset is undersampled just by its sparseness would be unrealistic. The reason  
 477 stems from a biological fact: a sizeable fraction of the maximum, potential links  
 478 that can be recorded among two distinct sets of species is simply unobservable,  
 479 irrespective of sampling effort (Jordano, 1987). In addition, sampling effort needs  
 480 to be explicitly gauged because of its potential influence on parameter estimates  
 481 for the network.

482 Missing links are a characteristic feature of all plant-animal interaction net-  
 483 works, and likely pervade other ecological interactions. Important natural history  
 484 details explain a fraction of them, resulting in unrealizable interactions (i.e., for-  
 485 bidden interactions) that define structural zeroes in the interaction matrices and



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486 contribute to their extreme sparseness. Sampling interactions is a way to monitor  
487 biodiversity beyond the simple enumeration of component species and to develop  
488 efficient and robust inventories of functional interactions. Yet no sampling theory  
489 for interactions is available. Focusing just on the realized interactions or treating  
490 missing interactions as the expected unique result of sampling bias would miss  
491 important components to understand how all sorts of interactions coevolve within  
492 complex webs of interdependence among species.

493 Contrary to species inventories, a sizable fraction of non-observed pairwise  
494 interactions cannot be sampled, due to biological constraints that forbid their  
495 occurrence. Moreover, recent implementations of inference methods for unobserved  
496 species (Chao *et al.*, 2015) or for individual-based data (Wells & O'Hara, 2012)  
497 can be combined with the forbidden link approach. They do not account either  
498 for the existence of these ecological constraints, but can help in estimating their  
499 relative importance, simply by the difference between the asymptotic estimate of  
500 interaction richness *in a robustly-sampled* assemblage and the maximum richness  
501  $I_{max}$  of interactions.

502 Ecological interactions provide the wireframe supporting the lives of species,  
503 and they also embed crucial ecosystem functions which are fundamental for sup-  
504 porting the Earth system. We still have a limited knowledge of the biodiversity  
505 of ecological interactions, and they are being lost (extinct) at a very fast pace,  
506 frequently preceding species extinctions (Valiente-Banuet *et al.*, 2014). We ur-  
507 gently need robust techniques to assess the completeness of ecological interactions  
508 networks because this knowledge will allow the identification of the minimal com-  
509 ponents of their ecological complexity that need to be restored to rebuild functional  
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526 **Data accessibility**

527 This review does not use new raw data, but includes some re-analyses of previously  
528 published material. All the original data supporting the paper, R code, supple-  
529 mentary figures, and summaries of analytical protocols is available at the author's  
530 GitHub repository ([https://github.com/pedroj/MS\\_Network-Sampling](https://github.com/pedroj/MS_Network-Sampling)), with  
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## 724 Figure captions

725 **Figure 1.** Sampling ecological interaction networks (e.g., plant-animal interac-  
 726 tions) usually focus on different types of subsampling the full network, yielding  
 727 submatrices  $\Delta[m, n]$  of the full interaction matrix  $\Delta$  with  $A$  and  $P$  animal and  
 728 plant species. a) all the potential plants interacting with a subset of the animals  
 729 (e.g., studying just the hummingbird-pollinated flower species in a community);  
 730 b) all the potential animal species interacting with a subset of the plant species  
 731 (e.g., studying the frugivore species feeding on figs *Ficus* in a community); and c)  
 732 sampling a subset of all the potential animal species interacting with a subset of all  
 733 the plant species (e.g., studying the plant-frugivore interactions of the rainforest  
 734 understory).

735

736 **Figure 2.** Sampling species interactions in natural communities. Suppose an  
 737 assemblage with  $A = 3$  animal species (red, species 1–3 with three, two, and 1  
 738 individuals, respectively) and  $P = 3$  plant species (green, species a–c with three  
 739 individuals each) (colored balls), sampled with increasing effort in steps 1 to 6  
 740 (panels). In Step 1 we record a total of three interactions (black lines) for animal  
 741 species 1 and plant species a and b, represented as two distinct pairwise interac-  
 742 tions:  $1 - a$  and  $1 - b$ . As we advance our sampling (panels 1 to 6, illustrating  
 743 e.g., additional sampling days) we record new distinct interactions. Note that we  
 744 actually sample and record interactions among individuals, yet we pool the data  
 745 across species to get a species by species interaction matrix. Few network analyses  
 746 have been carried out on individual data (e.g., Dupont *et al.*, 2014). Above and  
 747 below each panel are the cumulative number of distinct species and interactions

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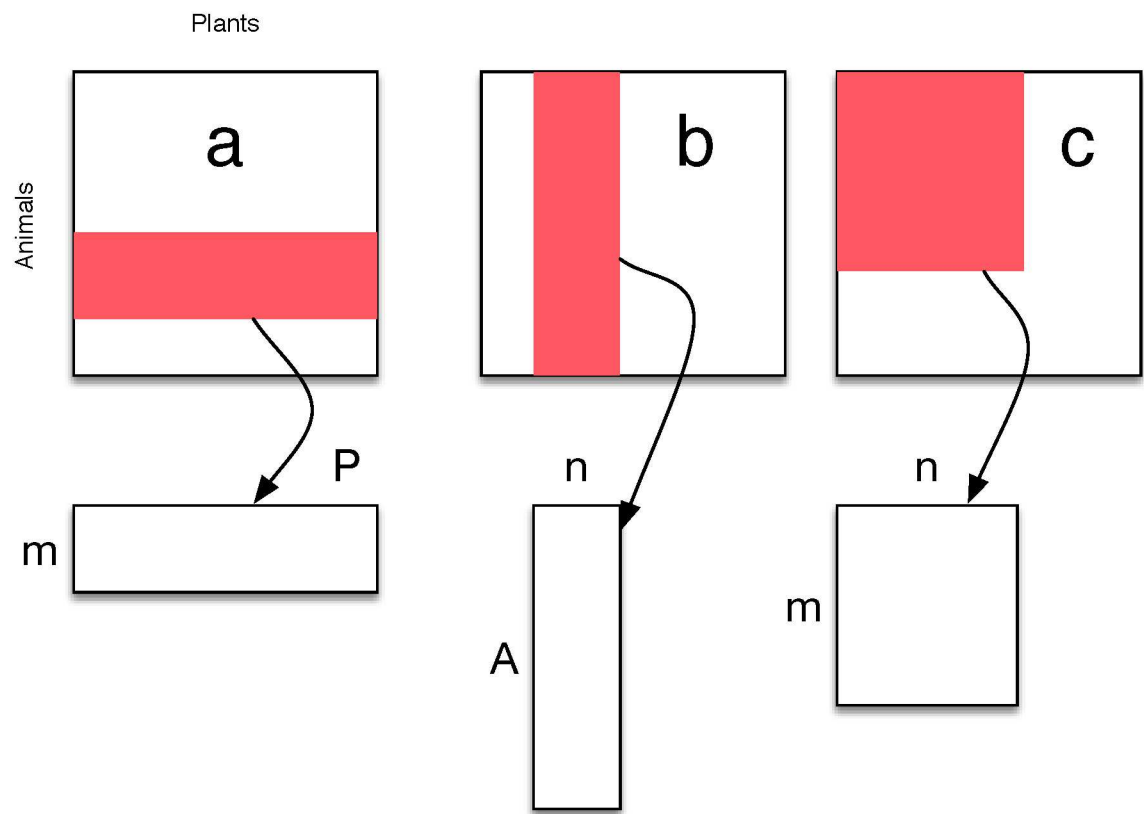
748 sampled, so that panel 6 illustrates the final network.

749

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750 **Figures**

Figure 1:



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Figure 2:

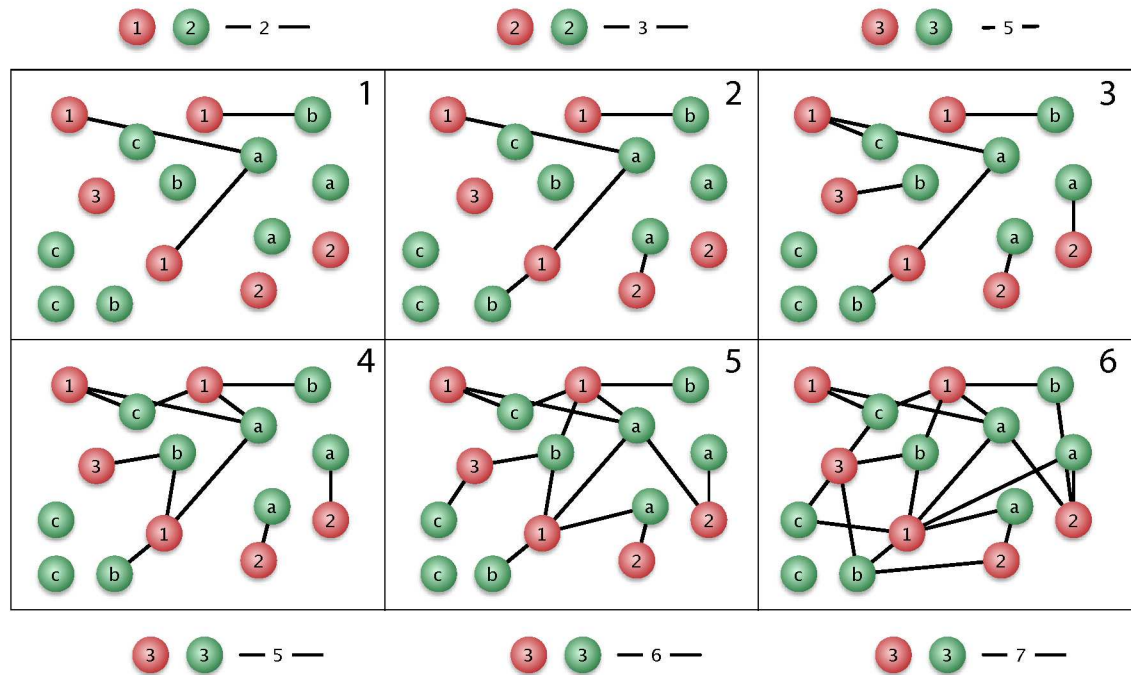


Table captions

**Table 1.** A taxonomy of link types for ecological interactions (Olesen *et al.* 2011).  $A_{obs}$ , number of animal species;  $P_{obs}$ , number of plant species;  $I_{obs}$ , number of observed links;  $FL$ , number of forbidden links; and  $ML$ , number of missing links. As natural scientists, our ultimate goal is to eliminate  $ML$  from the equation  $FL = AP - I - ML$ , which probably is not feasible given logistic sampling limitations. When we, during our study, estimate  $ML$  to be negligible, we cease observing and estimate  $I$  and  $FL$ .

**Table 2.** Frequencies of different type of forbidden links in natural plant-animal interaction assemblages.  $AP$ , maximum potential links,  $I_{max}$ ;  $I$ , number of observed links;  $UL$ , number of unobserved links;  $FL$ , number of forbidden links;  $FL_P$ , phenology constraints;  $FL_S$ , size restrictions;  $FL_A$ , accessibility constraints;  $FL_O$ , other types of restrictions;  $ML$ , unknown causes (missing links). Relative frequencies (in parentheses) calculated over  $I_{max}$  for  $I$  and  $UL$ ; those for  $FL$ , and  $ML$ , over  $UL$ ; for all forbidden links types ( $FL_P$ ,  $FL_S$ ,  $FL_A$ ,  $FL_O$ ), calculated over  $FL$ . Data for Hato Ratón and Nava Correhuelas include interactions with bird and mammal frugivores. References, from left to right: Olesen *et al.* 2008; Olesen & Myrthue unpubl.; Snow & Snow 1972 and Jordano *et al.* 2006; Vizentin-Bugoni *et al.* 2014; Jordano *et al.* 2009; Olesen *et al.* 2011.

**Table 3.** A vectorized interaction matrix corresponding to the sampling shown in Figure 2. All the possible distinct pairwise interactions (cells of the adjacency matrix) between animal species ( $A$ ) 1, 2, and 3 and plant species ( $P$ )  $a$ ,  $b$  and  $c$



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775 can be potentially recorded in any of the six sampling periods (panels in figure  
776 1). For each sampling period,  $N$  is the cumulative number of records and  $I$  is  
777 the cumulative number of distinct pairwise interactions recorded (1's in the binary  
778 adjacency matrix).

779

780 **Table 4.** Sampling statistics for three plant-animal interaction networks (Olesen  
781 *et al.* 2011). Symbols as in Table 1;  $N$ , number of records;  $Chao1$  and  $ACE$  are  
782 asymptotic estimators for the number of distinct pairwise interactions  $I$  (Hortal  
783 *et al.* 2006), and their standard errors;  $C$ , sample coverage for rare interactions  
784 (Chao & Jost 2012). Scaled asymptotic estimators and their confidence intervals  
785 ( $CI$ ) were calculated by weighting  $Chao1$  and  $ACE$  with the observed frequencies  
786 of forbidden links. Data for Hato Ratón and Nava Correhuelas include only plant-  
787 bird interactions.

788

## 789 Tables

Table 1:

Link type	Formulation	Definition
Potential links	$I_{max} = A_{obs}P_{obs}$	Size of observed network matrix, i.e. maximum number of potentially observable interactions; $A_{obs}$ and $P_{obs}$ , numbers of interacting animal and plant species, respectively. These might be below the real numbers of animal and plant species, $A_{est}$ and $P_{est}$ .
Observed links	$I_{obs}$	Total number of observed links in the network given a sufficient sampling effort. Number of ones in the adjacency matrix.
True links	$I_{est}$	Total number of links in the network given a sufficient sampling effort; expected for the augmented $A_{est}P_{est}$ matrix.
Unobserved links	$UL = I_{max} - I_{obs}$	Number of zeroes in the adjacency matrix.
True unobserved links	$UL* = I_{max} - I_{obs}$	Number of zeroes in the augmented adjacency matrix that, eventually, includes unobserved species.
Forbidden links	$FL$	Number of links, which remain unobserved because of linkage constraints, irrespectively of sufficient sampling effort.
Observed Missing links	$ML = A_{obs}P_{obs} - I_{obs} - FL$	Number of links, which may exist in nature but need more sampling effort and/or additional sampling methods to be observed.
True Missing links	$ML* = A_{est}P_{est} - I_{est} - FL$	Number of links, which may exist in nature but need more sampling effort and/or additional sampling methods to be observed. Augments $ML$ for the $A_{est}P_{est}$ matrix.

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Table 2:

Link type	Pollination			Seed dispersal		
	Zackenberg	Grundvad	Arima Valley	Sta. Virginia	Hato Ratón	Nava Correhuelas
$I_{max}$	1891	646	522	423	320	900
$I$	384 (0.2031)	212 (0.3282)	185 (0.3544)	86 (0.2033)	151 (0.4719)	181 (0.2011)
$UL$	1507 (0.7969)	434 (0.6718)	337 (0.6456)	337 (0.7967)	169 (0.5281)	719 (0.7989)
$FL$	530 (0.3517)	107 (0.2465)	218 (0.6469)	260 (0.7715)	118 (0.6982)	320 (0.4451)
$FL_P$	530 (1.0000)	94 (0.8785)	... (...)	120 (0.4615)	67 (0.5678)	195 (0.6094)
$FL_S$	... (...)	8 (0.0748)	30 (0.1376)	140 (0.5385)	31 (0.2627)	46 (0.1438)
$FL_A$	... (...)	5 (0.0467)	150 (0.6881) <sup>a</sup>	... (...)	20 (0.1695)	79 (0.2469)
$FL_O$	... (...)	... (...)	38 (0.1743) <sup>b</sup>	... (...)	... (...)	... (...)
$ML$	977 (0.6483)	327 (0.7535)	119 (0.3531)	77 (0.2285)	51 (0.3018)	399 (0.5549)

<sup>a</sup>, Lack of accessibility due to habitat uncoupling, i.e., canopy-foraging species vs. understory species.

<sup>b</sup>, Colour restrictions, and reward per flower too small relative to the size of the bird.

Dots indicate no data available for the  $FL$  type.

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Table 3:

Interaction	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
1-a	2	2	2	3	3	5
1-b	1	2	2	3	4	4
1-c	0	0	1	2	2	2
2-a	0	1	2	2	3	3
2-b	0	0	0	0	0	2
2-c	0	0	0	0	0	0
3-a	0	0	0	0	0	0
3-b	0	0	1	1	1	2
3-c	0	0	0	0	1	2
<i>N</i>	3	5	8	11	14	20
<i>I</i>	2	3	5	5	6	7

Table 4:

	Hato Ratón	Nava Correhuelas	Zackenberg
<i>A</i>	17	33	65
<i>P</i>	16	25	31
<i>I<sub>max</sub></i>	272	825	1891
<i>N</i>	3340	8378	1245
<i>I</i>	151	181	268
<i>Chao1</i>	263.1 ± 70.9	231.4 ± 14.2	509.6 ± 54.7
<i>ACE</i>	240.3 ± 8.9	241.3 ± 7.9	566.1 ± 14.8
<i>% unobserved<sup>a</sup></i>	8.33	15.38	47.80

<sup>a</sup>, estimated with library Jade (R Core Development Team 2010, Chao *et al.* 2015)