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Using metabarcoding to ask if easily collected soil and leaf-litter samples can be used as a general biodiversity indicator

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ABSTRACT

The targeted sequencing of taxonomically informative genetic markers, sometimes known as metabarcoding, allows eukaryote biodiversity to be measured rapidly, cheaply, comprehensively, repeatedly, and verifiably. Metabarcoding helps to remove the taxonomic impediment, which refers to the great logistical difficulties of describing and identifying species, and thus promises to improve our ability to detect and respond to changes in the natural environment. Now, sampling has become a rate-limiting step in biodiversity measurement, and in an effort to reduce turnaround time, we use arthropod samples from southern China and Vietnam to ask whether soil, leaf litter, and aboveground samples provide similar ecological information. A soil or leaf-litter sample can be collected in minutes, whereas an aboveground sample, such as from Malaise traps or canopy fogging, can require days to set up and run, during which time they are subject to theft, damage, and deliberate contamination. Here we show that while the taxonomic compositions of soil and leaf-litter samples are very different from aboveground samples, both types of samples provide similar ecological information, in terms of ranking sites by species richness and differentiating sites by beta diversity. In fact, leaf-litter samples appear to be as or more powerful than Malaise-trap and canopy-fogging samples at detecting habitat differences. We propose that metabarcoded leaf-litter and soil samples be widely tested as a candidate method for rapid environmental monitoring in terrestrial ecosystems.

1. Introduction

The environmental monitoring and indicator literature collectively calls for the *efficient* measurement of *total* biodiversity (or a substantial non-biased sample) (Andelman and Fagan, 2000; Cushman et al., 2010; Dolman et al., 2012; Edwards et al., 2014; Knight et al., 2008, 2010; Lindenmayer and Likens, 2010; Newton, 2011; Nicholson et al., 2012; Possingham et al., 2012; Stuart et al., 2010), which seem to be contradictory goals, given the infamous "taxonomic impediment" (Ebach et al., 2011). The impediment refers to the great logistical difficulties of describing and identifying species.

However, metabarcoding technology (Baird and Hajibabaei, 2012; Bik et al., 2012; Ji et al., 2013; Taberlet et al., 2012) is a strong candidate for achieving both goals. Metabarcoding combines DNA taxonomy with high-throughput DNA sequencing to identify mass samples of eukaryotes. Amplicons of species-discriminating 'barcode' genes from soil, water, or collections of organisms reveal the presence and, more noisily, the frequencies of species of fungi, plants, and animals (Bienert et al., 2012; Bohmann et al., 2014;

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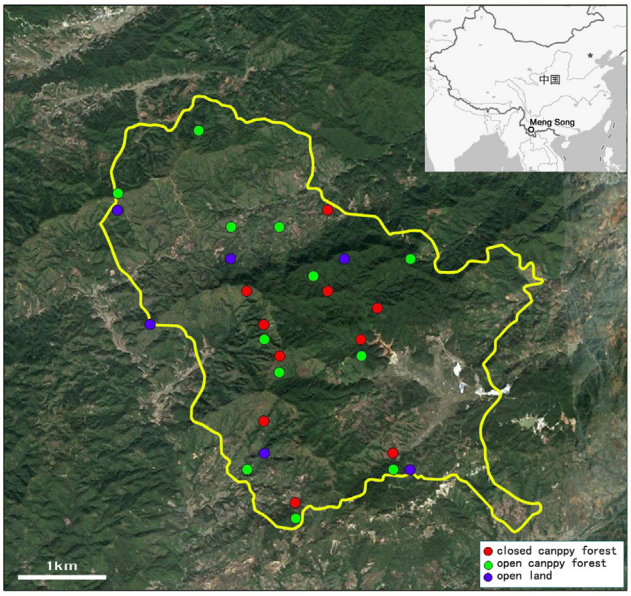
Calvignac-Spencer et al., 2013; Fonseca et al., 2010; Hajibabaei et al., 2012; Hiiesalu et al., 2012; Ovaskainen et al., 2010; Thomsen et al., 2012; Yoccoz et al., 2012; Yu et al., 2012) and can recover ecological information in the form of alpha- and beta-diversity estimates (Fonseca et al., 2010; Hiiesalu et al., 2012; Yoccoz et al., 2012; Yu et al., 2012). Importantly, such collections are auditable, because sites can be sampled by independent parties and analyzed by certified entities following a standard protocol. Metabarcode datasets are also taxonomically more comprehensive, many times quicker to produce, and less reliant on taxonomic expertise (Baird and Hajibabaei, 2012; Bik et al., 2012; Ji et al., 2013; Taberlet et al., 2012). Finally, Ji et al. (2013) have shown that metabarcode and standard (=morphologically identified species) biodiversity datasets result in very similar management decisions for monitoring, restoration ecology, and systematic conservation planning. In sum, metabarcoding promises reliable, verifiable, taxonomically comprehensive, and cost-effective biodiversity measurement.

Despite this promise, however, considerable work remains before metabarcoding can be considered for widespread adoption. In this paper, we focus on a seemingly simple but very useful question. Is it possible to substitute ground-level (soil or leaf litter) samples for aboveground samples when conducting biodiversity surveys (Ibáñez et al., 2012; Taberlet et al., 2012)?

An important advantage of a ground-level sample is that it can be cheaply collected in minutes (followed by processing in the lab), whereas an aboveground sample, such as from canopy fogging or various traps (e.g. Malaise, flight-intercept, pitfall, light, and baited) are more expensive and can require days and multiple personnel, and traps are subject to theft, damage, vandalism, and deliberate contamination. For instance, we have observed colleagues losing Malaise traps to elephants and to children, leading to unbalanced sampling effort. The need to retrieve aboveground traps incurs extra field expenses and logistical complications. As a result, if an environmental-certification organization were to judge, say, whether a set-aside area were truly maintaining biodiversity (following the potential example of Ewers et al. (2011) for oil-palm plantations), aboveground traps would be problematic, because such samples could be deliberately adulterated by local managers (Newton, 2011; Meijaard & Sheil, 2012). In short, one-shot, ground-level samples, coupled with metabarcoding to overcome the taxonomic impediment, could let us squeeze out costs and possibilities for fraud in our monitoring data, thus accelerating environmental measurement.

We have previously designed pipelines to metabarcode arthropod biodiversity from aboveground samples (Yu et al., 2012; Ji et al., 2013) and from soil and leaf litter (Yang et al., 2013). We now use these pipelines to compare soil and leaf-litter samples with Malaise-trap samples in southern China (Meng Song) and to compare leaf-litter samples with canopy-fogging and morphologically identified spider samples in central Vietnam (Vu Quang and Bach Ma). The samples were deliberately placed over a gradient of anthropogenic disturbance, and we ask if the different sample types all differentiate habitats in the same way.

A. Meng Song, China



B. Vu Quang (top) and Bach Ma (bottom), Vietnam.

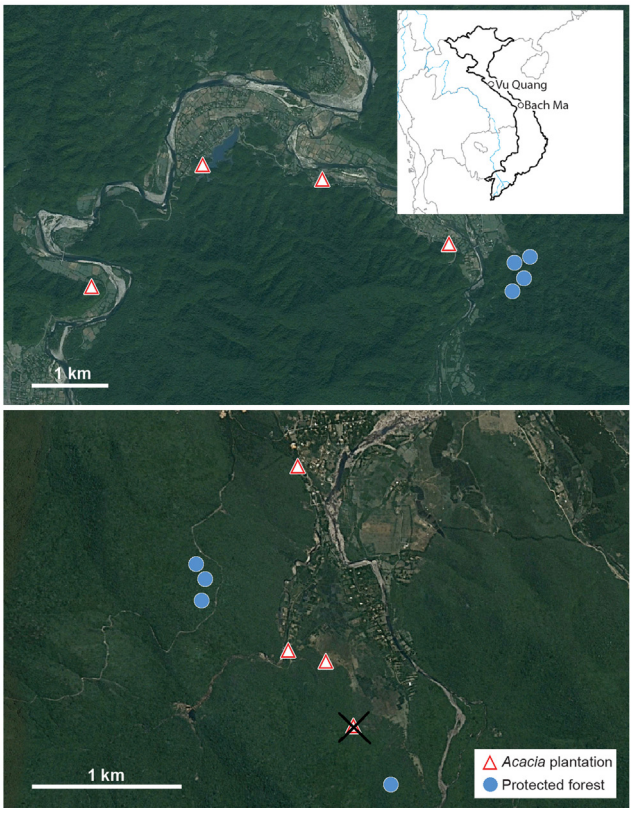


Fig. 1. Sampling maps. (A) Meng Song, China. Twenty-eight total samples are divided amongst 10 closed canopy forest sites, 12 open canopy forest sites, and 6 open land sites. (B) Bach Ma, Vietnam. (C) Vu Quang, Vietnam. Each Vietnam location was sampled in 8 sites: 4 *Acacia* plantations and 4 protected forests. Protected-forest sampling sites were clustered, due to limited accessibility. The sample marked with a black X indicates the sample that was omitted for contamination (Bach Ma – *Acacia*). (A) Meng Song, China.

2. Materials and methods

2.1. Meng Song, China

Meng Song is a village administrative unit in the Xishuangbanna prefecture of southern Yunnan, China (Fig. 1A, 21.5° N 100.5° E). The landscape includes part of the Bulong Nature Reserve, which is composed of seasonal montane rain forest and broadleaf evergreen forest (Zhu et al., 2005). The main cash crop is tea, which is grown as ~3 m tall understory trees in plots within thinned portions of the nature reserve (essentially, a kind of 'shade tea,' analogous to shade coffee). Additionally, monoculture tea plantations have previously been cultivated on cleared land that consists of rows of tea shrubs in a matrix of grass, plus scattered individual trees and shrubs. In this landscape, 28 1-ha quadrats were established for a large biodiversity census project (Making Mekong Connected; Xu

et al., 2013). Quadrat sites were selected using a stratified (by disturbance class and location), random approach. A 500 × 500 grid of points was generated over the entire ~100 km^2 landscape, and each point was classified to one of the three habitat disturbance classes: Closed canopy forest, Open canopy forest (with the understory tea trees), and Open land (=monoculture tea plantation). Next, after ground-truthing, the study area was divided into 16 equal-area units. Out of the 16 units, 12 units were randomly selected, and one closed canopy and one open canopy forest point were selected from each unit. Open land points were randomly selected from every second unit, since we expected this disturbance class to be more self-similar (Beckschäfer et al., 2013). Each point became the SW corner of a 100 m × 100 m quadrat. These quadrats were surveyed for vegetation, soil and other components of biodiversity between Apr 2010 and Mar 2012.

2.1.1. Malaise-trap samples

In Meng Song, insects were trapped over two periods separated by 6–7 months (Sep–Nov 2010 and Apr–Jun 2011). Every quadrat was trapped in each period using five Malaise traps over 6 days. One trap was placed in the center and one in each corner of the quadrat, as in the number five on a die. The bottles were half-filled with 99.9% ethanol, and after collection, the alcohol was replaced and the samples stored in a −4 °C freezer until transport to Kunming city, where the samples were stored in a −20 °C freezer for 1 month before being metabarcoded.

2.1.2. Soil and leaf-litter samples

Each quadrat was subdivided into nine 10 m radius subquadrats. A soil (0–15 cm) and a leaf-litter sample were taken from each subquadrat and then pooled within each quadrat. For leaf litter, a 1 m × 1 m sample per subquadrat was sifted through a 10 mm litter-sifter for 1 min to remove whole leaves and larger impurities (Yang et al., 2013). To avoid cross-contamination, the litter-sifter was soaked in bleach for >40 min and then rinsed between quadrats. For soil, approximately 100 g of mineral soil from 10 cm below the surface was sampled using a trowel and spoon. In each subquadrat, soil was combined from four points, which were approximately 2 m north, east, south, and west from the subquadrat's center point. To avoid cross contamination between quadrats, the trowel and spoon were flamed in alcohol. All samples were stored in 99.9% ethanol and in a −4 °C freezer until transport to Kunming city where the samples were stored in a −20 °C freezer for 1 month before being metabarcoded.

2.2. Vu Quang and Bach Ma, Vietnam

Vu Quang (Ha Tinh province) and Bach Ma (Mang Cà Province) are national parks in Vietnam (Fig. 1B). In and near both parks, four sites in protected forest and four in *Acacia* plantation were sampled. The region of Vu Quang sampled is near a network of villages including Kim Quang, Huong Quang, and Tung Quang. Villagers exploit Man Chan forest, which is part of the park, for natural resources. Among the settlements are scattered, small *Acacia* plantations (plot areas: VQ1: 1405 m^2; VQ3: 11,085 m^2; VQ7: 9785 m^2; VQ8: 2105 m^2, estimated visually using Google Earth Pro), which are grown for wood and pulp. Sample sites were located at approximately 18.3° N 105.4° E and range in elevation from 30 to 75 m; sampling occurred between 16 and 22 April 2011.

The region of Bach Ma sampled straddles the park border. The area inside the park is moderately sloped while the area outside the park is flat. Outside the park is a large semi-continuous *Acacia* monoculture (plot areas: BM8: 80,770 m^2 and BM1, 4, 6: all part of one semi-continuous area of 110,045 m^2, estimated visually using Google Earth Pro). We encountered no signs of natural resource exploitation inside the park. Sample sites were located at approximately 16.2° N 107.8° E; sites within the park range in elevation between 125 and 190 m; sites outside the park are below 70 m; sampling occurred between 9 and 13 April 2011.

2.2.1. Fogging samples

At each sample site, 30 1-m^2 sheets were arranged on the forest floor or among understory vegetation. We used an IZ-Fog device (http://foggers.co.kr) to deliver approximately 2 L of Permethrin 50 EC (Hockley International LTD) diluted 10:1 with diesel. Arthropods were allowed to accumulate on the sheets for 1–2 h before collecting into sample bags containing 70% ethanol. Half the sheets per sample site were pooled for metabarcoding; specimens from the remaining sheets were sorted to morphospecies, and morphospecies concepts were later checked with DNA barcode sequencing (J.A. Miller, data not shown). Due to limited taxonomic resources, only the Vu Quang spiders were sorted to morphospecies (n = 8 samples). This is a clear example of how the taxonomic impediment can vitiate biodiversity monitoring.

2.2.2. Leaf litter samples

For leaf litter samples, 5 m^2 of leaf litter were gathered into a sifter and agitated. The concentrate was then collected and left to dry in a Winkler litter extractor (www.entowinkler.at) for 36–48 h. A Winkler litter extractor is a tent-like apparatus with hanging mesh bags that can be filled with concentrated leaf litter, and a jar of alcohol at the bottom (Shaw and Ozanne, 2011). As the litter dries, arthropods leave the bags and fall into the 70% ethanol below.

All samples for metabarcoding were stored in the original 70% ethanol at room temperature until transferred to Kunming city where the samples were stored in a −20 °C freezer for 1 month before being metabarcoded.

2.3. Metabarcode protocols: DNA extraction, PCR, and 454 pyrosequencing

2.3.1. COI amplicons from Malaise-trap and Canopy-fogging samples

We prepared the aboveground samples by using two legs from all specimens equal to or larger than a mosquito and whole bodies of everything smaller, adding 4 mL Qiagen ATL buffer (Hilden, Germany) (20 mg/mL proteinase K = 9:1) per 1.0 g of sample, homogenizing with sterile 0.25-inch ceramic spheres in a FastPrep-24 system (MP Biomedicals, Santa Ana, CA) set on 5 m/s for 10 s at room temperature, incubating overnight at 56 °C, and using 10% of the lysed solution for genomic DNA extraction with Qiagen DNeasy Blood & Tissue Kits, using no more than 900 μL per spin column. The quantity and quality of purified DNA was assessed using the Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). Samples were PCR amplified using the degenerate primers *Fol-degen-for* 5′-TCNACNAAYCAYAARRAYATYGG-3′ and *Fol-degen-rev* 5′-TANACYTCNGGRTGNCCRAARAAYCA-3′, which amplify a 658-base-pair portion of the mitochondrial cytochrome c oxidase subunit I (COI) gene. The standard Roche A-adaptor and a unique 10 bp MID tag for each sample were attached to the forward primer. Each sample was amplified in three independent reactions and pooled. PCRs were performed in 20 μL reaction volumes containing 2 μL of 10× buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 μM each primer, 0.6 U HotStart Taq DNA polymerase (TaKaRa Biosystems, Ohtsu, Japan), and approximately 60 ng of pooled genomic DNA. We used a touchdown thermocycling profile of 95 °C for 2 min; 11 cycles of 95 °C for 15 s; 51 °C for 30 s; 72 °C for 3 min, decreasing the annealing temperature by 1 °C every cycle; then 17 cycles of 95 °C for 15 s, 41 °C for 30 s, 72 °C for 3 min, and a final extension of 72 °C for 10 min We used non-proofreading Taq and fewer, longer cycles to reduce chimera production (Lenz and Becker, 2008; Yu

et al., 2012). For pyrosequencing, PCR products were gel-purified by using a Qiagen QIAquick PCR purification kit, quantified by using the Quant-iT PicoGreen dsDNA Assay kit (Invitrogen), pooled and A-amplicon-sequenced on a Roche GS FLX at the Kunming Institute of Zoology. Further details are provided in Yu et al. (2012).

2.3.2. 18S amplicons from soil and leaf-litter samples

We prepared the ground-level samples by using 1-mm and then 63-μm cylindrical steel sieves to separate soil fauna from larger soil and leaf-litter particles. For soil samples, we also used water decantation in 500 mL graduated cylinders to separate soil fauna, which float, from heavier sand particles (Creer et al., 2010; Yang et al., 2013). Decantation cannot be used to separate fauna from leaf-litter particles, which both float. The floating portion of each soil sample and the leaf-litter samples were homogenized in a FastPrep-24 system using steel beads at 45 m/s for 20 s (Yang et al., 2013). The PowerSoil DNA isolation kit (MO BIO Laboratories, Carlsbad, CA, USA) was used to extract DNA from 0.25 g per sample. Following Fonseca et al. (2010), we used *SSU_F04* (5′-GCTT GTCTCAAAGATTAAGCC-3′) and *SSU_R22* (5′-GCCTGCTGCCTTCCTT GGA-3′) primers, which amplify a 450 bp portion of the 18S nuclear small subunit (nSSU) ribosomal RNA (rRNA) gene. The standard Roche A-adaptor and a unique 10 bp MID tag for each sample were attached to the forward primer. PCRs were carried out in 10 μL reaction volumes containing 0.8 μL of dNTP mixture (1.25 mmol L^{-1} each base), 5.65 μL of distilled water, 0.05 μL of Taq DNA polymerase (TaKaRa Biosystems, Ohtsu, Japan), 1.0 μL of 10× PCR buffer (100 mmol L^{-1} Tris–HCl (pH 8.3), 500 mmol L^{-1} KCl, 15 mmol L^{-1} $MgCl_2$), 0.4 μL of each primer (20 μmol L^{-1}), 0.5 μL DMSO, 0.2 μL BSA and 1.0 μL DNA template. Non-proofreading Taq and fewer, longer cycles were used to reduce chimera production. Thermal cycling conditions for amplification of the 18S sequences were 2 min at 95 °C, followed by 30 cycles of 15 s at 95 °C, 30 s at 57 °C and 3 min at 72 °C, and a final elongation stage of 10 min at 72 °C. Each sample was amplified three times independently, and the products were pooled for sequencing (30 μL total per sample).

2.3.3. 454 pyrosequencing

All PCR products were gel-purified using a QIAquick PCR Purification Kit (Qiagen GmbH, Hilden, Germany), quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA, USA), pooled and A-amplicon-sequenced on a Roche GS FLX '454' System. For one of the Meng Song quadrats, we separately PCR-amplified and sequenced the subquadrats, resulting in a total of 72 PCR products (9 subquadrats within one quadrat + 27 pooled quadrats = 36 for soil and another 36 for leaf litter). Our reason was to see if deeper sequencing (9 separate subquadrats) would reveal qualitatively different results, and we found that it did not (C. Yang, data not shown), so we merged the sequence data from these subquadrats into one quadrat for all downstream processing. From Vietnam, we sequenced 32 PCR products (16 leaf litter + 16 canopy fogging).

The 72 Meng Song samples were sequenced on four 1/8 regions, producing 289,206 raw reads and 196,977 post-quality-control (QC) reads (mean read length 343 bp). The 16 Vietnam leaf litter samples were sequenced on one 1/8 region, producing 100,710 raw reads and 67,647 post-QC reads (260 bp). The 16 Vietnam canopy samples were sequenced on three 1/8 regions, producing 224,922 raw reads and 110,940 post-QC reads (248 bp).

2.4. Bioinformatic extraction of operational taxonomic units (OTUs) from raw sequence data

For the COI data, we used Yu et al.'s (2012) pipeline to denoise and cluster the reads into Operational Taxonomic Units (OTUs). Quality control: Header sequences and low-quality reads were removed from the raw output in the QIIME 1.5.0 environment (split_libraries.py: -l 100 -L 700 -H 9 -M 2 -b 10) (Caporaso et al., 2010b). Denoising and chimera removal: PyNAST 2.7 (Caporaso et al., 2010a) was used to align reads against a high-quality, aligned dataset of Arthropoda sequences (Yu et al., 2012) at a minimum similarity of 60%, and sequences that failed to align were removed. The remaining sequences were clustered at 99% similarity with USEARCH 5.2 (Edgar, 2010), a consensus sequence was chosen for each cluster, and the UCHIME function was used to perform de novo chimera detection and removal. A clustering step is required for chimera detection because chimeric reads are expected to be rare and thus should belong to small clusters only. The final denoising step used MACSE 0.9b1 (Ranwez et al., 2011), which aligns at the amino-acid level to high-quality reference sequences and uses any stop codons in COI to infer frameshift mutations caused by homopolymers. We removed any sequences <100 bp. OTU-picking and Taxonomic assignment: Sequences were clustered into 96%-similarity OTUs using CROP 1.3 (Hao et al., 2011). OTUs were assigned taxonomies using SAP 1.0.12 (Munch et al., 2008), keeping only taxonomic levels for which the posterior probability was >80%. OTUs containing only one read were removed. Computations were performed on a combination of Apple iMacs and a Linux computing cluster at the University of East Anglia (rscs.uea.ac.uk/high-performance-computing, accessed 18 May 2013). Sequence data will be deposited at datadryad.org and in GENBANK's Short Read Archive.

For the 18S data, we followed Yang et al.'s (2013) USEARCH/CROP pipeline, which mostly follows the COI pipeline above but assigns taxonomies to OTUs by BLASTing against the SILVA rDNA database release 108 (Pruesse et al., 2007) at 0.001 maximum *e*-value. We discovered that one leaf-litter sample from Bach Ma was comprised almost entirely of insects from canopy fogging traps, and we therefore deemed the sample to be mislabelled or contaminated, and we omitted it from downstream analysis.

The end result was three OTU X sample tables from Meng Song: soil (sieved and bead-beaten, $n = 28$), leaf litter (bead-beaten, $n = 28$) and Malaise traps ($n = 28$), and three tables from Vietnam: leaf litter (Winkler funnel, $n = 16$), canopy fogging ($n = 16$), and spiders (morphology, $n = 8$).

2.5. Statistical analyses

Analyses were performed using *R* 2.15.1 (R Core Team, 2012), *vegan* 2.0 (Oksanen, 2011), *QIIME* (Caporaso et al., 2010b) and *mvabund* 3.7.0 (Warton et al., 2011). To compare species richness, we used incidence-coverage estimators, which were calculated with *vegan*'s *specpool* function. The number of reads per OTU correlates noisily with OTU biomass and abundance (Amend et al., 2010; Yu et al., 2012). We thus converted read number to presence/absence using vegan's *decostand* function (Gentleman et al., 2012; Oksanen, 2011), and we used only OTUs that had been assigned to Arthropoda, as our COI primers were designed for this phylum. To visualize the effects of environmental treatment levels on community compositions, we used Principal Coordinates Analysis (PCoA) ordination of 1-Sørensen-Dice dissimilarity matrices (Faith et al., 1987), which were created in QIIME. To compare PCoA ordinations between datasets, we used vegan's *protest* Procrustes correlation tests. For hypothesis testing, we used *mvabund* to test the effects of environmental predictors on community composition. *mvabund* is a multivariate implementation of generalized linear models, and, unlike dissimilarity-matrix-based methods, *mvabund* does not confound location with dispersion effects, which can inflate type 1 and 2 errors (Wang et al., 2012; Warton et al., 2011). We corrected for multiple tests using the *p.adjust* (*method* = "*fdr*") function in *R*'s base package (Benjamini and Hochberg, 1995; Zuur et al., 2009).

Table 1
Taxonomic assignment of OTUs (operational taxonomic units) for above- and below-ground samples. Soil and leaf litter samples were assigned taxonomies using the SILVA 108 18S database, and Malaise traps and canopy fogging samples were assigned taxonomies using SAP 1.0.12. See Section 2 for assignment details. At each taxonomic level, the number of OTUs assigned to that level is reported. All OTU frequencies below the level of Metazoa are calculated relative to all metazoan OTUs. Some small taxonomic groups are omitted for clarity, so subgroup frequencies do not add up to the higher group frequency (e.g. Insecta + Collembola OTUs < Hexapoda OTUs). (A) Meng Song, China. (B) Vu Quang and Bach Ma, Vietnam. Insecta OTUs dominate the Malaise traps and canopy fogging samples, whereas Arachnida are more frequent in the soil and leaf litter samples, as expected. Note also that a higher proportion of eukaryote OTUs in the Winkler-funneled leaf litter samples (B. Vietnam, 83.5%) are assigned to Metazoa, compared to purely bead-beaten leaf litter samples (A. China, 51.6%), suggesting that metazoan-specific 18S primers nonetheless amplify many non-metazoans when applied to DNA extracted directly from soil or leaf litter.

A. Meng Song, China

Sample and Marker	Eukaryota	Metazoa	Arthropoda				Nematoda	Annelida
Soil (sieved and bead-beaten) 18S	545 OTUs (90.1% of Total)	269 (44.5% of Eukaryota)	163 (60.6% of Metazoa)				47 (17.5% of Metazoa)	24 (8.9% of Metazoa)
			Arachnida 64 (23.8%)	Hexapoda 55 (20.4%)		Myriapoda 28 (10.4%)		
				Insecta 36 (13.4%)	Collembola 10 (3.7%)			
Leaf litter (bead-beaten) 18S	652 (94.4% of Total)	318 (55.1% of Eukaryota)	220 (69.2 % of Metazoa)				35 (11.0% of Metazoa)	20 (6.3% of Metazoa)
			Arachnida 60 (18.9%)	Hexapoda 103 (32.4%)		Myriapoda 37 (11.6%)		
				Insecta 92 (28.9%)	Collembola 5 (1.6%)			
Malaise traps COI	2641 (95.2% of Total)	2634 (99.7% of Eukaryota)	2591 (98.4% of Metazoa)				25 (0.9% of Metazoa)	5 (0.2% of Metazoa)
			Arachnida 130 (4.9%)	Hexapoda 2436 (92.5%)		Myriapoda 0		
				Insecta 2346 (89.1%)	Collembola 86 (3.3%)			

B. Vu Quang and Bach Ma, Vietnam

Sample and Marker	Eukaryota	Metazoa	Arthropoda				Nematoda	Annelida
Leaf litter (Winkler funnel) 18S	322 OTUs (91.2% of Total)	269 (83.5% of Eukaryota)	223 (82.9% of Metazoa)				20 (7.4% of Metazoa)	12 (4.5% of Metazoa)
			Arachnida 55 (20.4%)	Hexapoda 131 (48.7%)		Myriapoda 28 (10.4%)		
				Insecta 120 (44.6%)	Collembola 11 (4.1%)			
Canopy fogging COI	2371 (72.8% of Total)	2326 (98.1% of Eukaryota)	2271 (97.6% of Metazoa)				1 (0.04% of Metazoa)	2 (0.08% of Metazoa)
			Arachnida 259 (11.1%)	Hexapoda 1927 (82.9%)		Myriapoda 4 (0.2%)		
				Insecta 1853 (79.7%)	Collembola 74 (3.2%)			

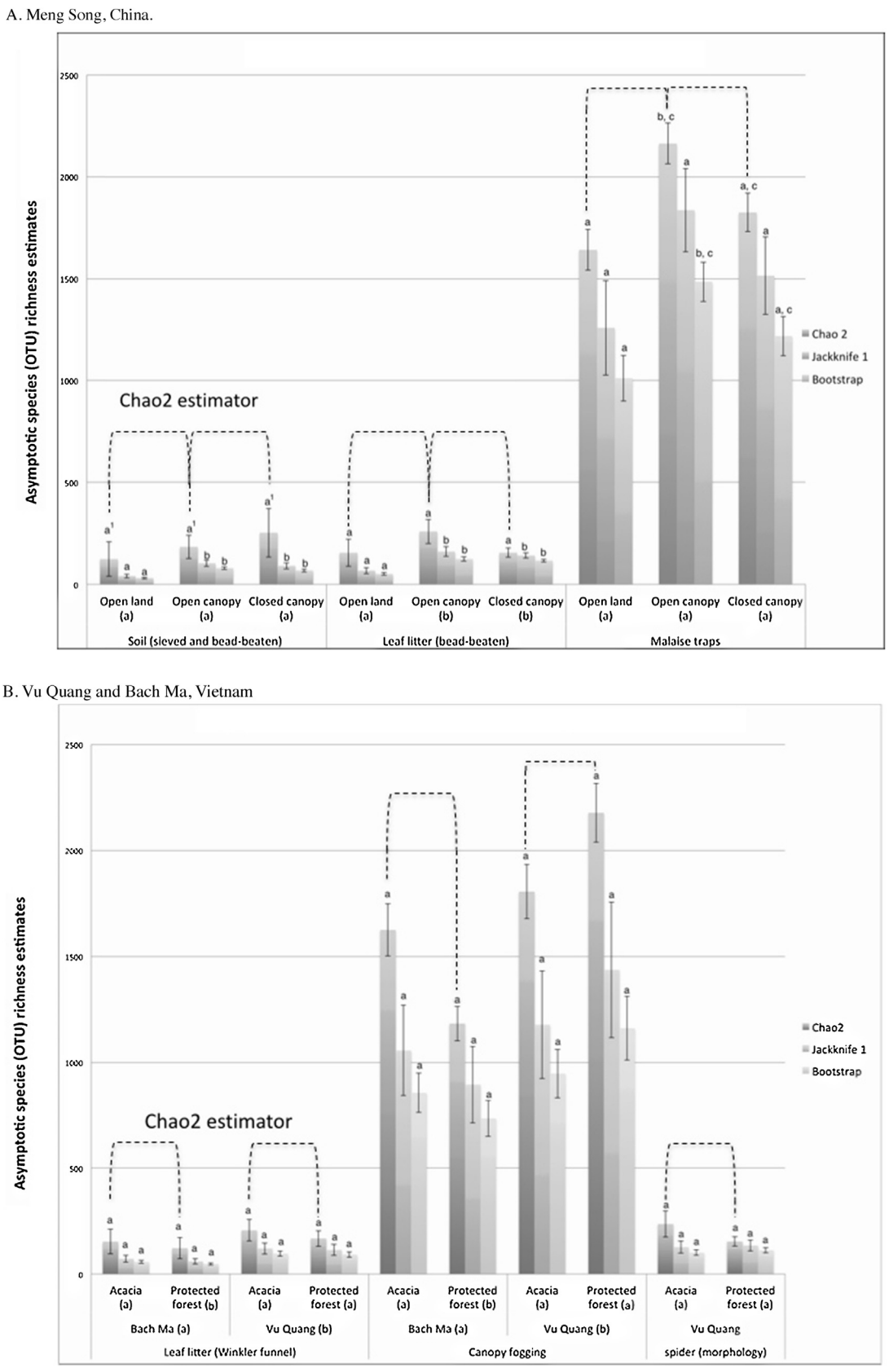


Fig. 2. Comparisons of asymptotic arthropod species (OTU) richness estimates (alpha diversity) and community composition (beta diversity) across three habitat types and three sample types. Superscripts above the vertical bars indicate whether habitat types differ significantly in species richness (function *specpool* in *vegan*, Welch's *t*-tests). Letters in parentheses in the bottom row of each figure indicate whether habitat types differ significantly in species composition (*mvabund*, generalized linear model, binomial errors). To aid visual comparison, the Chao2 species richness estimates for the three habitats have been connected with a dashed line within each sample type. Significance values are Bonferroni-corrected for three simultaneous tests per sample type in China, using the *p.adjust* (*method* = "*fdr*") function in *vegan* and a Type 1 significance threshold of $p = 0.05$ after correction. We report corrected *p*-values here. (A) Meng Song, China. *Species richness*: for soil and leaf litter samples, species richness is higher in the two

Example *QIIME* and *R* scripts for the pipelines and analyses conducted here are available in (Yang et al., 2013; Yu et al., 2012; Ji et al., 2013).

3. Results

3.1. Taxonomic compositions

In Table 1, we present the higher-level taxonomies that were assigned to the OTUs from the soil, leaf-litter, Malaise-trap, and canopy-fogging samples (OTU tables with their respective sequences are provided in Supplementary materials). A higher proportion of eukaryote OTUs in the Winkler-funneled leaf litter samples (Table 1B. Vietnam, 83.5%) were assigned to the Metazoa, relative to the bead-beaten leaf litter samples (Table 1A. Meng Song, 55.1%), showing that the metazoan-specific 18S primers still amplify many non-metazoans, mostly green plants and fungi, when applied to DNA extracted directly from soil or leaf litter.

Within the metazoan OTUs, the taxonomic compositions of the metabarcode datasets were consistent with the microhabitats from which the samples were collected (Table 1). Soil and leaf-litter OTUs were broadly distributed across the Arthropoda, Annelida, and Nematoda. Within the Arthropoda, Arachnida made up around 20% of the metazoan OTUs. In contrast, Malaise-trap and canopy-fogging OTUs were dominated by the Insecta (80–90% of metazoan OTUs).

We can now ask our original motivating question: do these very different samples from different microhabitats return the same ecological information? That is, do ground-level and aboveground metabarcoded samples differentiate sites and habitat classes in the same way?

3.2. Species richness

For all datasets, each treatment level (here, habitat type) was sampled multiple times, making it possible to use incidence-coverage estimators (Gotelli and Colwell, 2011) to extrapolate total species richness per treatment level. We used Chao2, Jackknife1, and Bootstrap from the *specpool* function in *vegan*.

3.2.1. Meng Song, China

In all three datasets (soil, leaf litter, and Malaise), observed species richness was lower in the open land habitat (Fig. 2A). In the soil and leaf-litter datasets, two of the three total-richness estimators (Jackknife1 and Bootstrap) found this difference to be statistically significant after correcting for three post hoc comparisons. In the Malaise-trap dataset, the confidence intervals for the estimators were larger, and only open-canopy forest was deemed to be significantly more species-rich than the open land habitat (Chao2 and Bootstrap estimators). We did not record any significant differences in species richness between the two forest habitats. In sum, both ground-level and aboveground datasets found forested habitats to be more species rich, and the ground-level datasets showed this more consistently.

3.2.2. Vu Quang and Bach Ma, Vietnam

All datasets (leaf litter, canopy fogging, and spider morphospecies) and all estimators within each dataset agreed that total species richness did not differ across forest type (*Acacia* vs. Protected forest) (Fig. 2B). In fact, observed species richness was sometimes (non-significantly) higher in *Acacia* forest than in neighboring protected forest sites.

3.3. Community composition

Here, we used the software *mvabund* to test whether community compositions change across habitats (and also across locations in Vietnam) in the same way for the different sample types.

3.3.1. Meng Song

Only leaf-litter samples separated open land from the two forest habitats. The two forest habitats were not differentiated by any of the sample types (bottom row of subscripts in parentheses in Fig. 2A). We also used Procrustes tests of PCoA ordinations to visualize and test for correlations between datasets (Hamady et al., 2010). The Malaise-trap ordination was significantly correlated with the leaf-litter ordination (Fig. 3A) but only marginally correlated with soil samples (Fig. 3B), although in both pairwise comparisons, the correlation coefficients were high ($r_{\text{malaise-leaf}} = 0.78$, $p = 0.001$, $r_{\text{malaise-soil}} = 0.87$, $p = 0.08$). In short, the Malaise-trap and ground-level datasets appear to contain similar ecological information about the differences in the three habitat types, despite being composed of very different species.

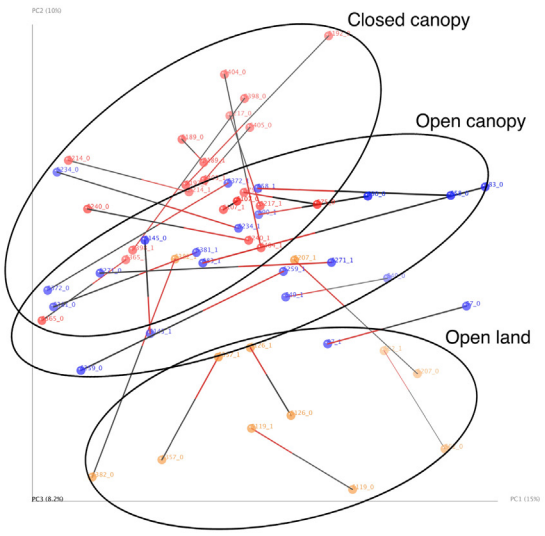
3.3.2. Vietnam

Both the leaf-litter and canopy-fogging samples separated the Bach Ma and Vu Quang locations (bottom row of subscripts in parentheses in Fig. 2B). There was also a significant location × habitat interaction effect. The leaf-litter and canopy-fogging samples both differentiated *Acacia* from Protected forest, but only did so in Bach Ma (second-to-bottom row of subscripts in parentheses in Fig. 2B). In contrast, in Vu Quang, *Acacia* and Protected forest community compositions were statistically (Fig. 2B) and visually (Fig. 3C) indistinguishable in all three datasets: leaf litter, canopy fogging, and spider morphospecies. Unfortunately, our lack of taxonomic resources means that we had no spider morphospecies data from Bach Ma to test if the spider dataset also detects a location × habitat effect.

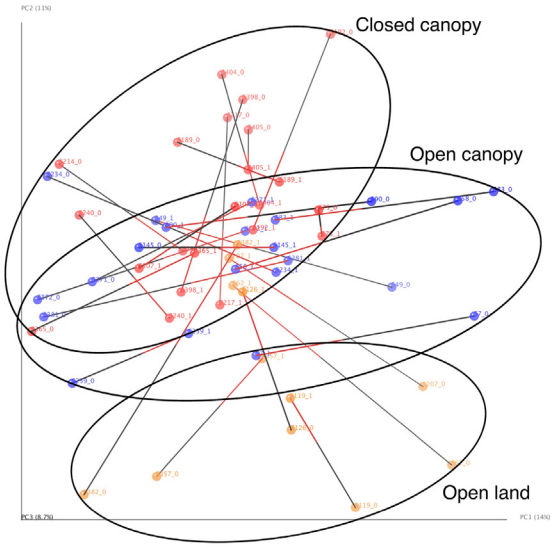
We also bioinformatically extracted the spider (Araneae) OTUs from the metabarcode datasets. PCoA + Procrustes correlation between the canopy-fogging Araneae OTUs and the spider morphospecies was non-significant (Fig. 3D), but leaf-litter Araneae

forested habitats (closed and open canopy) than in the open land habitat, with the difference being statistically significant for two of the richness estimators, Jackknife1 (all $p < 0.02$) and Bootstrap (all $p < 0.001$). Malaise trap species richness is also weakly higher in the two forest habitats. Only open canopy forest has statistically significantly higher richness than open land habitat, for two of the richness estimators (Chao2 and Bootstrap, all $p < 0.01$). *Community composition*: only leaf litter samples differentiate open land habitat from the two forest habitats (*mvabund*, Malaise traps, df = 2, deviance = 3457.71, $p = 0.34$; soil, df = 2, deviance = 189.35, $p = 0.31$; leaf litter, df = 2, deviance = 402.4, $p = 0.045$). The two forest habitat communities, open canopy and closed canopy, are not themselves differentiated by any of the sample types (*mvabund*, Malaise traps, df = 1, deviance = 1633.22, $p = 0.27$; soil, df = 1, deviance = 79.13, $p = 0.26$; leaf litter, df = 1, deviance = 183.29, $p = 0.08$). (B) Vu Quang and Bach Ma, Vietnam. *Species richness*: species richness is not significantly different between *Acacia* and protected forest habitats for any of the three estimators, nor for any of the three sample types (leaf litter, canopy fogging, and spiders). *Community composition*: the leaf litter and canopy fogging samples both differentiate Bach Ma from Vu Quang (*mvabund*, canopy fogging, deviance = 2968.6, df = 13, $p = 0.005$; leaf litter, deviance = 152.2 df = 13, $p = 0.017$). Only canopy fogging samples differentiate *Acacia* from protected forest, and only in Bach Ma (*mvabund*, location:habitat interaction effect, df = 11, deviance = 659.4, $p = 0.0017$; Bach Ma only habitat main effect, deviance = 1753.1, df = 5, $p = 0.023$; Vu Quang, $p = 0.51$). And leaf litter samples also show indications that *Acacia* and protected forest habitats are compositionally different in Bach Ma, the habitat effect is significant (*mvabund*, location:habitat interaction effect, df = 11, deviance = 39.63, $p = 0.026$; habitat main effect in reduced model, df = 12, deviance = 93.57, $p = 0.25$). Consistent with metabarcoding samples, the spider samples also do not differentiate *Acacia* and protected forest habitats in Vu Quang (*mvabund*, df = 6, deviance = 110.33, $p = 0.24$). The spider dataset cannot be used to test for habitat differences between Vu Quang and Bach Ma because the Bach Ma samples were not processed. [1]Species richness superscripts indicate pairwise comparisons for each extrapolation method separately (Chao2, Jackknife1, and Bootstrap). (B) Vu Quang and Bach Ma, Vietnam.

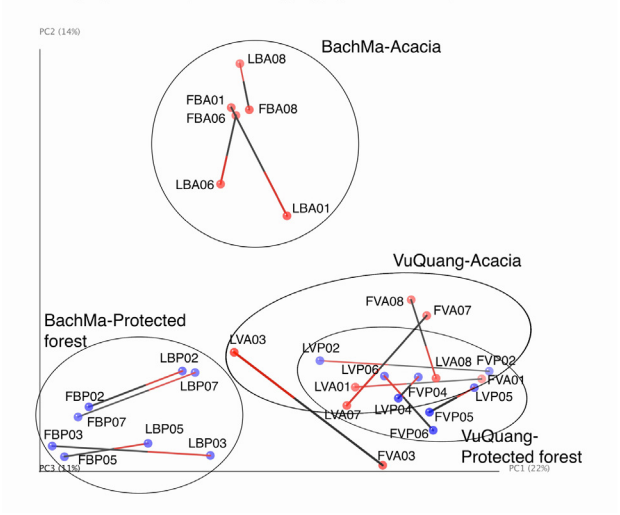
A. Meng Song, China. Malaise traps vs. Leaf litter samples.



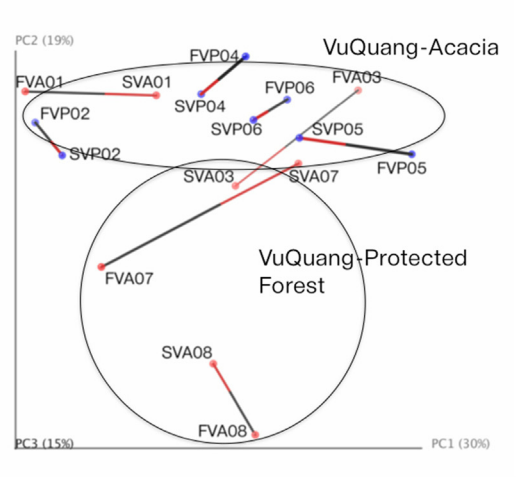
B. Meng Song, China. Malaise traps vs Soil samples.



C. Vu Quang and Bach Ma, Vietnam. Canopy fogging vs. Leaf litter samples.



D. Vu Quang, Vietnam. Canopy fogging vs. Spider (morphology) samples.



E. Vu Quang, Vietnam. Leaf litter vs. Spider (morphology) samples.

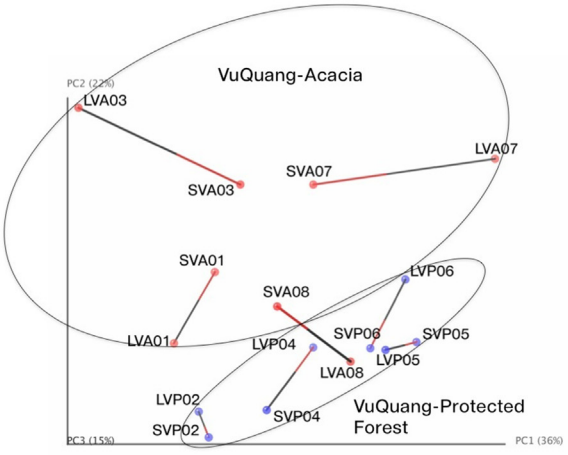


Fig. 3. Principal coordinate analysis (PCoA) ordinations, pairwise compared with Procrustes tests between sample types (soil, leaf litter, Malaise traps, and canopy fogging). Line segments connect paired samples, and ovals are used to help visualize compositional differences amongst habitats and localities. (A) Meng Song, China. Malaise traps vs. leaf litter ($r = 0.78$, $p = 0.003$). (B) Meng Song, China, Malaise traps vs. soil. (Procrustes $r = 0.87$, $p = 0.107$). Red = closed canopy, blue = open canopy, and yellow = open land. (C) Vu Quang, Vietnam, canopy fogging vs. leaf litter (Procrustes $r = 0.48$, $p = 0.0002$). (D) Vu Quang, Vietnam. Canopy fogging vs. spider (Procrustes $r = 0.54$, $p = 0.13$). (E) Vietnam. Leaf litter vs. spider (Procrustes $r = 0.48$, $p = 0.049$). L = leaf litter, F = fogging, S = spiders, B = Bach Ma, V = Vu Quang, A = *Acacia*, P = protected forest. The key result is that in the China and Vietnam studies, leaf litter and aboveground samples (Malaise traps and Canopy fogging, respectively) differentiate sites similarly. (A) Meng Song, China. B. Meng

OTUs and spider morphospecies were significantly correlated (Fig. 3E).

4. Discussion

We show that metabarcoding technology (Baird and Hajibabaei, 2012; Bik et al., 2012; Ji et al., 2013; Taberlet et al., 2012) could recover biodiversity data from whole collections of arthropods in samples of soil, leaf litter, Winkler-funneled leaf litter, Malaise traps, and canopy fogging. Ground-level samples, from which 18S was amplified, were dominated by OTUs assigned to arachnids, insects, myriapods, nematodes, and annelids. Aboveground samples, from which COI was amplified, were mostly insect OTUs (Table 1).

We caution that the higher prevalence of nematodes and annelids in the ground-level samples could reasonably be attributed to the different genetic markers used; the 18S primers were designed to amplify across the Metazoa, whereas our COI primers are only known to amplify successfully across the Arthropoda (Ji et al., 2013; Yu et al., 2012). (Our COI primers cannot be used to amplify from soil and leaf-litter samples because >99% of returned OTUs are bacterial [Yang et al., 2013].) Regardless, the taxonomic compositions of the metabarcode datasets are consistent with the microhabitats from which the samples were collected. Soil and leaf-litter microhabitats are indeed highly species-rich in spiders, mites, centipedes, millipedes, roundworms, and ringed worms (Fierer et al., 2012; Ji et al., 2013; Roger-Estrade et al., 2010; Thiele-Bruhn et al., 2012), whereas canopy-fogging and Malaise-trap samples do capture mostly insects.

4.1. Species richness

In Meng Song, soil and leaf-litter samples consistently found that both forest habitats were more species rich than was the open-land habitat, and Malaise-trap samples also found the two forest habitats to be more species rich than is open-land, but did so less consistently.

In Vietnam, all three sample classes, including the spider morphospecies dataset, concurred in finding no significant difference in richness between *Acacia* and Protected forest habitats.

4.2. Community composition

Ground-level and aboveground datasets also differentiated habitats similarly (Figs. 2 and 3), especially in Vietnam.

In Meng Song, leaf-litter samples significantly differentiated the two forest habitats from the open-land habitat (Fig. 2A). Soil and Malaise-trap samples did not do so significantly. However, the significant Procrustes correlation between the leaf-litter and Malaise-trap ordinations (Fig. 3B) suggests that Malaise-trap data are just less statistically powerful for differentiating these habitats. In other words, the discrepancy between the Procrustes and *mvabund* analyses is due to the different questions asked. In Procrustes tests, we ask only if community compositions vary in the same way across samples. In *mvabund* tests, we ask if an exogenous environmental predictor variable can explain some of that variance. Also, unlike standard community analyses that are based on distance matrices, *mvabund* avoids ascribing significant effects to environmental variables simply due to heteroscedasticity in the dataset (Warton et al., 2011), meaning that *mvabund* tends to be conservative in this respect.

In Vietnam, the leaf-litter and canopy-fogging samples both exhibited the same location X habitat interaction effect, separating the two locations of Bach Ma and Vu Quang and also separating *Acacia* from protected forest habitats in Bach Ma only (Figs. 2B and 3C). The spider morphospecies dataset concurred with the metabarcode datasets in finding no significant difference between habitats in Vu Quang (Fig. 2B). This appears to be a reasonable result, because the *Acacia* forests in Vu Quang are small plots (see Section 2) embedded within a much larger natural-forest habitat, so we expect considerable dispersal of arthropods between *Acacia* and protected forest habitats in Vu Quang ('spillover,' Koh, 2008; Lucey and Hill, 2012). Dispersal might also explain the lack of difference in species richness between habitats (Fig. 2B) even though tropical tree plantations generally are known to support low species richness (Lugo, 1992). In contrast, in Bach Ma, the *Acacia* plantations are ~7–80 times larger than those in Vu Quang, and we should therefore expect more habitat differentiation, as we observed (Fig. 2B).

4.3. Potential advantages of leaf litter as an environmental indicator

Our Meng Song results suggest that leaf litter might be more ecologically informative than soil and Malaise-trap samples, since leaf-litter samples more consistently showed that the two forest habitats are more species rich and are compositionally different from open-land habitats (Figs. 2A and 3A). We speculate that the Malaise-trap samples did not differentiate forest from open land habitats because flying arthropods move more easily between the two (Koh, 2008; Lucey and Hill, 2012). In Vietnam, the leaf-litter samples matched the canopy-fogging samples in detecting the habitat × location interaction effect (Figs. 2B and 3C).

It is possible that leaf-litter samples, being at the interface of belowground and aboveground communities, end up integrating ecological information from both. To date, the leaf-litter microhabitat has posed a severe taxonomic impediment (Graça, 2001; Henderson and Walker, 2009), but metabarcoding technology could unlock this information. Our results also show that using Winkler (and, we presume, Berlese) funnels to filter leaf-litter samples will result in a greater proportion of metazoan OTUs when metabarcoding (Table 1A vs. B). Whether this additional processing is worth the extra time and cost depends on particular situations.

Finally, an important advantage of a ground-level sample is that it can be cheaply collected in minutes (followed by processing in the lab), whereas an aboveground sample can require multiple collection days, leaving traps subject to theft, damage, vandalism, and deliberate contamination. Alternatively, if canopy-fogging is used, costs are higher.

As for processing costs, Ji et al. (2013) estimated a cost range of US$240–415 per sample for metabarcode samples, using the Roche GS FLX '454' sequencer. Illumina sequencers are considerably cheaper to run and can now produce full, 658-bp COI barcodes (Liu et al., 2013), so future costs are likely to be near the low end of that range, plus a profit margin should this technology eventually become commercially available. As for processing times, Ji et al. (2013) found that the "active workload" (lab + computer-facing work) of metabarcoding was approximately one quarter that of visually sorting indicator taxa to morphospecies, where the taxa ranged from moths to carabid beetles to spiders to ants. For the microscopic taxa typical of ground-level samples (Table 1), the time advantage of metabarcoding is certainly many times greater.

However, note that the increased detection power of metabarcoding requires greater efforts to guard against sample

Song, China. Malaise traps vs. soil samples. (C) Vu Quang and Bach Ma, Vietnam. Canopy fogging vs. leaf litter samples. (D) Vu Quang, Vietnam. Canopy fogging vs. spider (morphology) samples. (E) Vu Quang, Vietnam. Leaf litter vs. spider (morphology) samples.

cross-contamination (e.g. our bleaching of the collection equipment), because PCR + next-generation sequencing is able to detect even small amounts of DNA (see Liu et al., 2013).

5. Conclusions

Our study tested the hypothesis that metabarcoding will allow ground-level samples to substitute for aboveground samples when characterizing biodiversity patterns (Pulleman et al., 2012). The result is that, although the two classes of metabarcode data, ground-level and aboveground, were composed of very different taxa, we still found that these two classes of data returned similar ecological information. That is, ground-level and aboveground metabarcoded samples differentiated sites and habitat classes in the same way, and, therefore, to answer our motivating question, it could indeed be possible to substitute ground-level (soil or leaf litter) samples for aboveground samples when conducting biodiversity surveys, making large efficiency gains possible at the sampling stage.

All sampling methods have their own biases and noise, and there exists no 'true' biodiversity dataset to test against. We can only compare different methods and look for (1) statistical concordance, and (2) similar policy conclusions. In this study, we tested for statistical concordance. What is needed now are case studies in which ground-level and aboveground samples are compared for their policy-level conclusions (Ji et al., 2013). For instance, do the two sample types produce similar systematic conservation plans, detect similar community trends after a restoration treatment or other environmental change, and/or return similar results when testing basic ecological hypotheses? As an example using only aboveground samples, Edwards et al. (2014) compared the indicator performance of metabarcoded Malaise-trap samples (and taxon subsets thereof) with conventionally identified datasets of birds, scavenging mammals, dung beetles, and leaf-litter ants. Seven subsets (birds, leaf-litter ants, dung beetles, plus metabarcoded beetles, Hymenoptera, flies, and true bugs) proved to be consistently good predictors of the response of the other taxa to logging and oil palm, in that they made similar recommendations for how to optimally protect species richness across a matrix of logged and unlogged tropical forest.

More generally, our results and the many others that are rapidly accumulating in the metabarcoding literature (reviews in Baird and Hajibabaei, 2012; Bik et al., 2012; Bohmann et al., 2013, 2014; Calvignac-Spencer et al., 2013; Ji et al., 2013; Taberlet et al., 2012) suggest that metabarcoding shows great promise in allowing us to move away from indicator taxa and toward the measurement of total biodiversity, thereby improving our ability to track and manage the natural environment (Breure et al., 2012; Dolman et al., 2012; Edwards et al., 2014; Lindenmayer and Likens, 2010; Newton, 2011; Nicholson et al., 2012). Using convenient taxonomic subsets can lead to inflated or out-of-date estimates of the biodiversity value of particular habitats if the taxa used are wide-ranging or have long generation times and thus respond slowly to environmental change (reviewed in Edwards et al., 2014).

High efficiency in biodiversity surveys is a fundamental requirement if ecosystems are to be managed over the spatial scale of landscapes, meaning thousands to tens of thousands of square kilometers at a time. This is to help achieve policy additionality (e.g. forest protection in one site does not merely lead to deforestation in a neighboring site), to cover large-scale processes, such as migration and the emergent effects of landscape complexity, and simply because it is more efficient. To be sure, it is remote-sensing technology that will be the primary mode by which large-scale monitoring will be achieved, but efficient sampling techniques, combined with metabarcoding, will be needed to interpret the high volume of earth-observation data that is anticipated (Lynch et al., 2013).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ecolind.2014.06.028.

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