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ABSTRACT

Little is known about distribution patterns of micrometazoan organisms at different spatial scales and the mechanisms driving these patterns across different environments. Here we explore the fine-scale structure of tardigrades in a high-elevation *Polylepis* forest in northern Ecuador. To investigate spatial patterns of tardigrade abundance, we collected samples from different bryophyte taxa (hosts) on the woodland floor. We identified some tardigrades to species, but most taxa were considered at the level of morphological operational taxonomic units. Tardigrade assemblages differed in composition between host taxa, with some tardigrade taxa associated more with certain hosts, which might relate to host architecture or chemistry. Tardigrade occupancy, richness and abundance varied considerably between samples, and we estimate that more than 50 samples are required to estimate tardigrade taxon richness in this forest habitat. Physical distance between samples was not related to similarity of composition, and it seems that fine-scale differences in environmental conditions (including the distribution of host bryophytes) is much more important in determining tardigrade composition. We conclude that standardised, comprehensive sampling of terrestrial tardigrades at fine scales is necessary before making broader comparisons at coarser geographical scales. Such sampling should account for the diversity of potential hosts, with sufficient replication to capture tardigrade diversity.

Introduction

One of the challenges facing contemporary ecology is understanding biodiversity patterns in microscopic animals [1]. Little is known about the distribution of these organisms over different spatial scales, or the mechanisms driving spatial patterns of abundance in different environments [2–4]. Whilst there are a number of apparently general, scale-related patterns in ecology, such as species-area and species-energy relationships [5–14], it is unclear how much such patterns apply to meiofauna – animals smaller than 2 mm [15]. Since community composition of macroorganisms is easier to describe than that of microscopic organisms, the majority of studies have focused on studying species diversity of such macroorganisms [16–18].

Despite being poorly known in many cases, it is clear that meiofauna can comprise a significant fraction of the biodiversity in many ecosystems and play important roles in ecosystem function, as part of trophic webs, and in energy and nutrient transfer [19–21]. However, despite their abundance and ubiquity, the roles of these organisms are often poorly defined. In fact, even the basic taxonomy of meiofauna and their spatial patterns of abundance remain incompletely known [15]. One of those overlooked groups is the phylum Tardigrada: hydrophilous micrometazoans, normally 50–1200 µm in length, and closely related to arthropods and onychophorans.

Tardigrades represent a convenient meiofaunal group for study. They are relatively abundant in terrestrial, freshwater and marine systems, and might be the most widely distributed invertebrates on Earth [22]. They are potentially interesting ecologically as they share a common evolutionary history with other multicellular animals but have similar environmental needs and biological characteristics to many unicellular organisms [23,24], and can be important in trophic networks (as predators, herbivores and detritivores [25]), and as components of overall biomass [26]. Their frequent ability to enter a dormancy stage provides them with the ability to survive desiccation, significant temperature variations and other extreme conditions [27–31]. In addition, although tardigrade studies are limited practically by processing time (associated with sorting and mounting any microscopic organisms), their taxonomy is relatively well documented and updated checklists taxa and associated keys are regularly published [32,33].

Information about tardigrade distribution patterns comes mostly from information found in taxonomic

descriptions, but also from sampling studies at various geographical scales [e.g. 34–38]. Some species are apparently observed in many different parts of the world [39], whilst others have only been reported from a single locality [40–42]. It is not clear whether this reflects genuine differences in distribution or merely results from insufficient material, although, in many cases, the latter appears likely. Very few studies have attempted to examine finer-scale tardigrade distribution patterns quantitatively within habitats [20,28,43–45], and how representative these are is unclear. One feature of tardigrades is that they are apparently very variable in abundance at fine spatial scales, which can result in patchy datasets with many samples containing few or no organisms [37]. Meyer [46] and Glime [47], emphasised the importance of pilot studies to determine appropriate sampling strategies in such cases, but few studies have done this, or systematically explored the pattern or its practical consequences in nature [46].

In general, it has been suggested that the distribution of animals of microscopic size is highly influenced by the interaction between macroenvironmental characteristics (climate, soil, etc.,) and micro environmental factors (vegetation, bryophytes and leaf litter). It has been widely proposed that tardigrade distribution is highly influenced by microhabitat conditions [20,43,48]. However, ecological studies at small scales are very limited [20,38] with most focusing on the impact of meso- and macro-scale factors [e.g. 34,49]. Although little is known about tardigrade habitat associations [20,43,44], it is commonly assumed that species-specific habitat patterns do exist in these animals [20,40–42,50]. However, many existing studies have concentrated their elevational variations over relatively large spatial scales [50–52] and very few have conducted quantitative sampling or statistical analyses to determine relationships between tardigrade species diversity, abundance and environmental factors [48]. In addition, despite the fact that most studies of tardigrade diversity have focussed on the fauna on mosses and lichens [47], few of these studies have explored the extent to which tardigrade taxa are host specific [43,46,53]. It is not known with certainty whether there is a specific epifaunal association with a particular kind of host, or if most taxa are relative generalists in this regard. Rarer tardigrades may, for example, be associated with specific hosts, but the extent to which this is the case remains unclear.

This study explores fine scale variation in tardigrade assemblages in an Andean *Polylepis* woodland. We explore whether different bryophyte hosts differ consistently in the species of tardigrade they support, whether there is spatial structure to tardigrade assemblages within a microhabitat type and attempt to estimate the number of samples required to obtain a complete picture of tardigrade diversity at the woodland scale. This is the first such detailed exploration of Andean tardigrades, and indeed one of the first to investigate such factors in these organisms anywhere in the world.

Methods

The study was carried out in a forest consisting almost entirely of trees of *Polylepis incana* Kunth, located at 3,575 m in the buffer zone of El Ángel Ecological Reserve, Carchi Province in northern Ecuador (Figure 1). *Polylepis* is the dominant tree genus in such habitats, where it plays a keystone role close to the Andean treeline [54]. These woodlands occur higher than any others, most commonly on mountain slopes, in deep canyons and ravines, and often in boulder fields or on steep rocky terrain [55,56]. The trees give shelter to several species of epiphytic vascular plants, mosses and lichens, as well as animals, including mammals and birds [55]. The study site experiences very little seasonality as it is close to the Equator, with humid conditions all year round. At the site, average soil surface temperatures ranged between 12–14 °C, but night-time temperatures fell to around 5 °C (Balbina Ramsay, *personal observations*, 2011). The site was relatively flat with organic soil, decaying wood and leaf litter; the forest floor was grazed by livestock and occasionally visited by tourists from a nearby hotel. Samples were collected in shaded areas, typical of this forest type.

On 13 August 2011, we sampled tardigrades living in bryophytes on the ground only. Additional bryophytes were present on the contorted trunks of the trees and on the branches and twigs of the canopy. However, the effective quantification of the complex three-dimensional structure of *Polylepis* forests and other pertinent environmental variables (*e.g.* substrate, temperature, pH) was not practical in this study. Without such work, the addition of trunk and canopy sampling would add much unexplainable noise to the composition data, so we restricted the study to the forest floor.

Within an area of 400 m^2 in the woodland core, we collected five replicate samples of approximately 4 cm^3 uncompressed volume from pure monospecific patches of five bryophyte species ("pure hosts"): *Leptodontium longicaule* Mitt., *Pleurozium schreberi* (Brid.) Mitt., *Thuidium delicatulum* (Hedw.) Schimp., *Zygodon nivalis* Hampe, and *Chiloscyphus latifolius* (Nees) J.J. Engel & R.M. Schust. The first four species are mosses and the final species is a liverwort. The growth form and structure of each of these bryophytes is shown in (Figure 2). We also collected 25 samples from an area of intimately mixed *Thuidium delicatulum* and *Pleurozium schreberi* ("mixed host") at 0.5 m intervals on a grid. In total, 50 samples were collected. No other species of bryophytes were growing on the
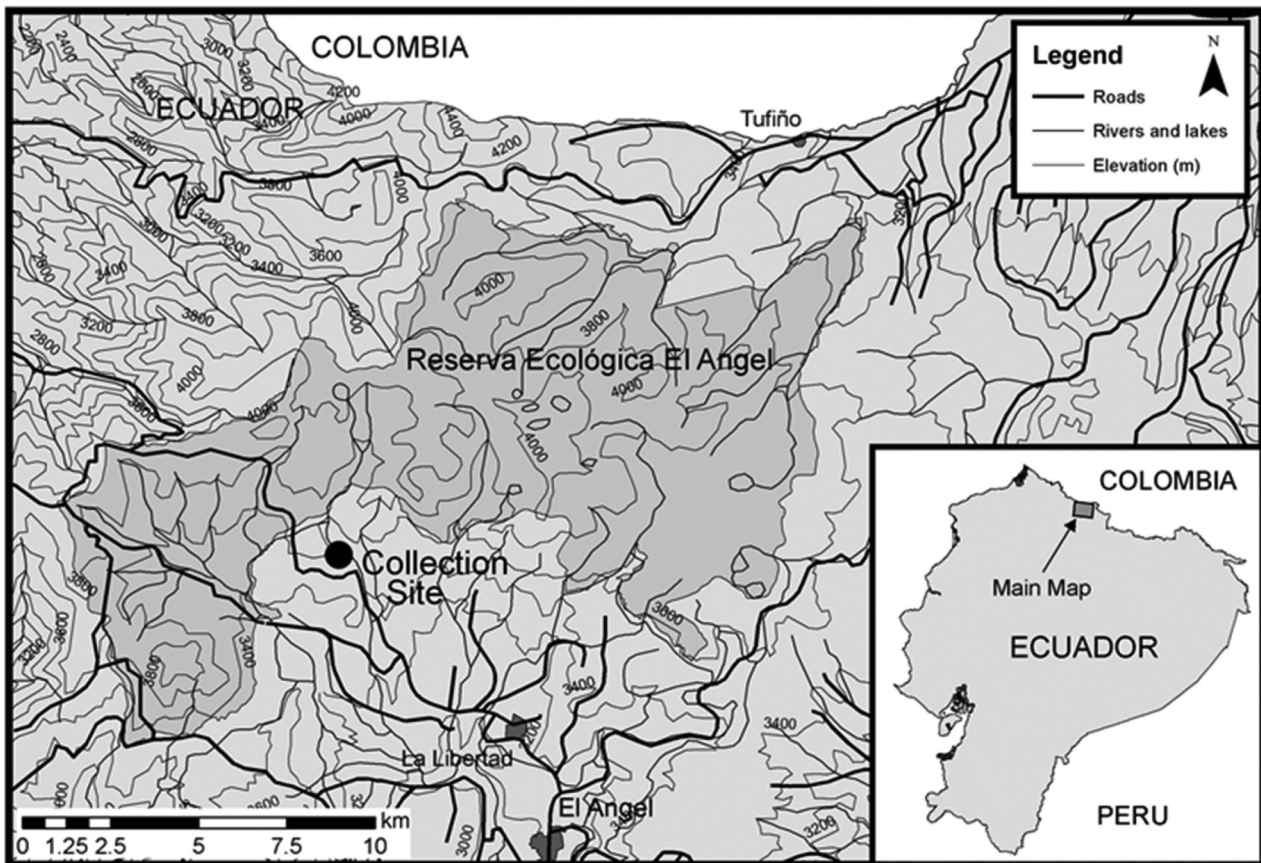
Figure 1. Location of the collection site from Reserva Ecológica El Ángel, Carchi Province in northern Ecuador at 3575 m elevation.

ground in the sampled area. Samples were air-dried in individual paper envelopes, and stored at 10–25 ºC until tardigrades were extracted.

In the laboratory, dried samples were rehydrated in tap water for 16–24 h. Rehydrated samples were shaken and passed through a 38 μm mesh sieve. Material retained by the sieve was searched for tardigrades using a Kyowa SDZ-PL stereoscopic microscope with 30–40x objectives (Kyowa, Japan). Tardigrades were mounted individually on microscope slides under cover slips in Hoyer's mounting medium. The identification of individual tardigrades was done to Operational Taxonomic Units (OTUs) according to its morphological characters with a Leica DMLB microscope with 40x and 100x objectives (the latter with immersion oil), using Guidetti and Bertolani [32], Marley et al. [57], and Degma [58]. All individuals were first identified to genus using observations of claw type, buccal apparatus and the number of placoids. For genera with several taxa present, individuals were classified into OTUs according to their morphological characteristics. For some genera, a three digit code was used representing, respectively, the number of macroplacoids, the number of microplacoids, and the presence of a septulum (e.g. *Adropion* sp. 311). Tardigrade taxa were also classified into four feeding groups according to Hallas and Yeates [59], and

personal observations of tardigrades by Balbina Ramsay and Nigel Marley (Figure 3). Individuals of the genus *Milnesium* were considered strict carnivores. The genera *Adropion*, *Diphascon*, *Echiniscus*, *Platicrista*, *Paramacrobiotus*, *Pilatobius*, *Mesocrista* and *Murrayon* were considered to be microbivores. Tardigrades with short, wide buccal tubes with strong stylets and large pharynxes were assumed to be omnivores, while the remaining tardigrades, with furca and apophyses, were considered herbivores.

Potential differences between host categories in overall tardigrade numbers, OTU richness and Shannon diversity were analysed using one-way General Linear Model (GLM ANOVA) or Kruskall-Wallis Tests, dependent on the outcome of a Shapiro-Wilks Test for normality – the non-parametric test was used for datasets that did not meet ANOVA's assumptions of normality. These statistical tests were carried out with R version 3.3.3 [60].

Species accumulation curves for tardigrade OTUs richness (*S*) for pure host and mixed host samples estimated the number of samples needed to fully characterize tardigrade communities. We used Estimate S (Version 9, R.K. Colwell, http://purl.oclc.org/esti mates) to plot the cumulative number of OTUs found as a function of sampling effort (species accumulation or rarefaction curves). For sample-based data, the
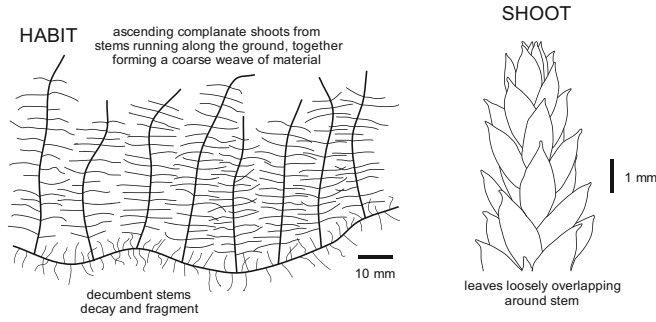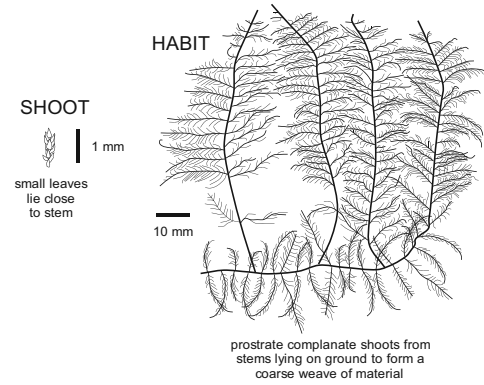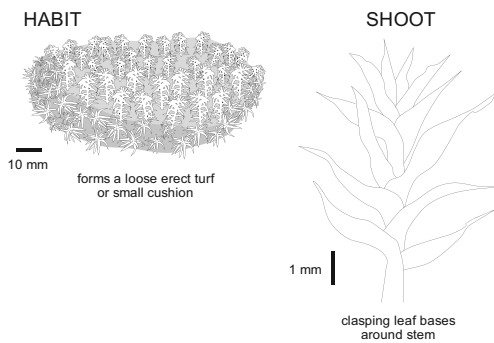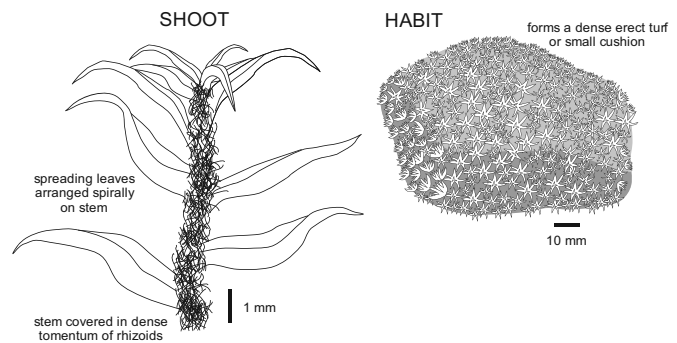
Figure 2. The habit and detailed morphology of the five bryophytes collected in this study: *Leptodontium longicaule, Pleurozium schreberi, Thuidium delicatulum, Zygodon nivalis* and *Chiloscyphus latifolius.*

estimator of asymptotic richness was Chao 2 [61,62]. The species accumulation curve was extrapolated to 50 samples (double the number of samples taken in each case, and the maximum extrapolation advised in the software user manual).

The OTU composition of samples was compared using non-metric Multidimensional Scaling (MDS) in performed with Primer 6 (Primer-e, Plymouth, UK), on square-root transformed OTU count data. The graphical output of this approach positions samples with similar composition close together and samples with more different composition further apart. Statistical differences in composition between host categories were determined by permutational ANOVA (PERMANOVA) using the PERMANOVA+ add-on to Primer 6. PERMANOVA is sensitive to differences in the dispersion of data [63] and so an additional test, when significant differences were identified by PERMANOVA, was carried out to identify any significant differences in dispersion between groups, using Primer 6's PERMDISP.

To determine whether OTU composition (measured as percentage similarity in tardigrade OTU composition of pairs of samples) could be predicted by physical distance between the samples, reduced major axis (RMA or Model II) regression was conducted in R using the package "lmodel2" on the mixed host samples, using a one tailed test [64].
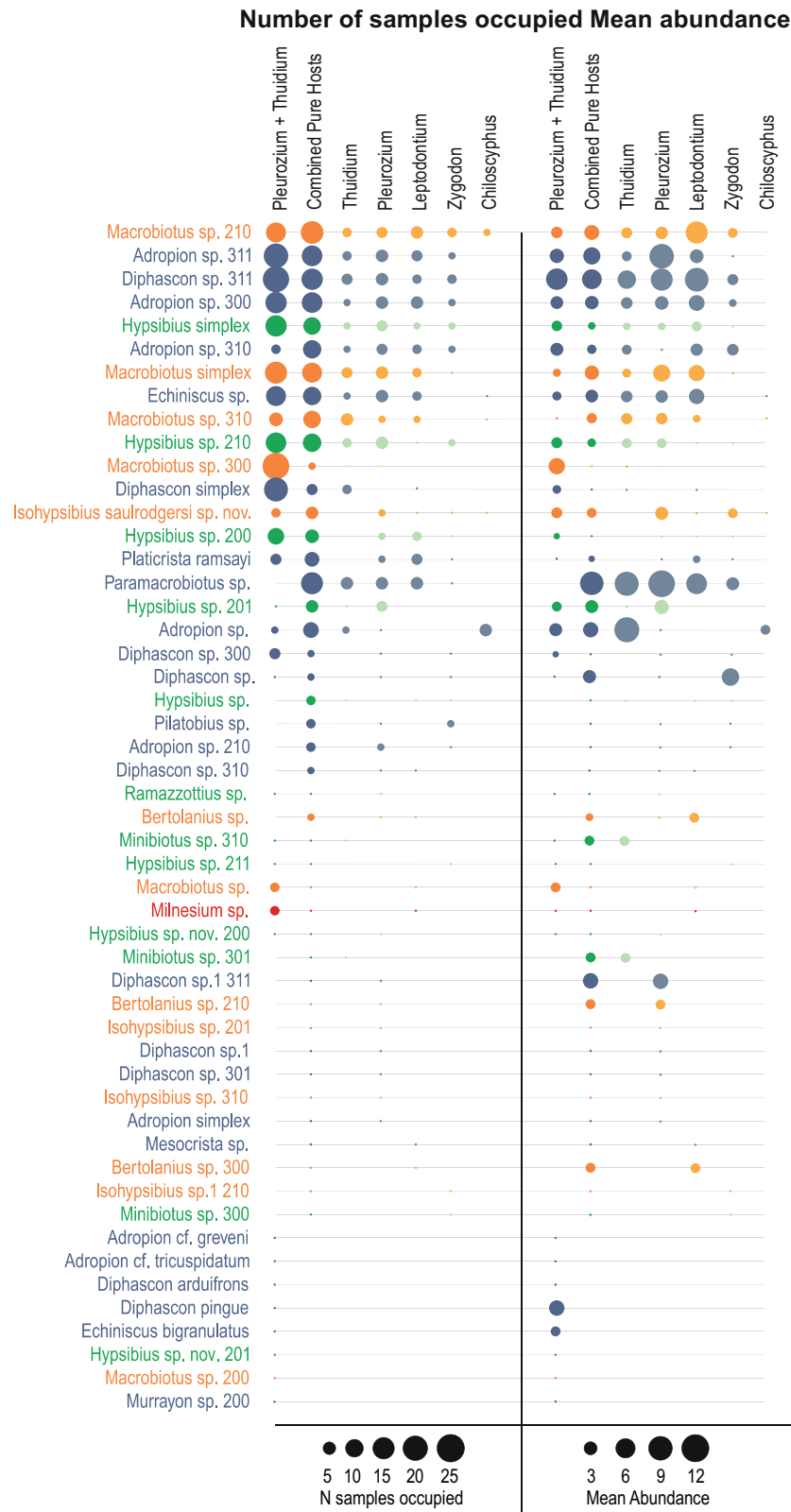
Figure 3. Tardigrade OTUs in 50 samples of bryophytes from a *Polylepis* woodland at 3575 m in Carchi Province, Ecuador. The area of the circles represents the number of samples occupied (left panel) or the mean abundance within the relevant samples (right panel), with a legend at the foot of each panel. Coloured circles represent a different tardigrade feeding habits, yellow for omnivore, blue for microbivore, green for herbivore and red for carnivore. OTUs named "cf." and "sp." followed by a number refer to recognizable morphospecies, some of which are new to science, and are to be described in the future. The "combined pure hosts" columns represents the tardigrades from all the pure host samples added together.

Results

Across all fifty samples (mixed and pure hosts combined), we identified 51 tardigrade OTUs (Figure 3). Some tardigrades found in this study represent new taxa (e.g. *Adropion* cf. *greveni*, *A.* cf. *tricuspidatum*, *Hypsibius* sp. nov. 200, *Hypsibius*. sp. nov. 201, *Isohypsibius saulrodgersi* sp. nov. and *Isophypsibius* sp. 1 210). *Macrobiotus* 210 is the only taxon present in all bryophyte species examined (pure and mixed). Some rare OTUs observed in this study occurred as single individuals, such as *Adropion* cf. *greveni* and *A.* cf. *tricuspidatum*.

Forty-three tardigrade OTUs, comprising 692 specimens, were found across the pure host samples (Figure 3). Individual samples contained 1–74 individuals and up to 16 OTUs. Eutardigrades outnumbered heterotardigrades in abundance (659 vs. 32 individuals) and taxon richness (31 vs. 1 taxa), with just one individual from the apotardigrades. Thirty-three OTUs were found across 648 specimens in the mixed host samples. Individual samples here contained 5–62 individuals and up to 17 OTUs. Eutardigrades again outnumbered heterotardigrades in abundance (620 vs. 25 individuals) and taxon richness (29 vs. 2 taxa), with three individuals from a single apotardigrade taxon. Across all the samples, there were 25 microbivore taxa, 13 omnivore taxa, 12 herbivore taxa and one strict carnivore taxon.

The abundant species were found consistently in most of the hosts (Figure 3). However, many species – even abundant ones – were not found in the *Chiloscyphus* host samples. Some species were also missing from the *Zygodon* host samples. The majority of taxa (61%) were relatively sparse in the samples, occurring in low numbers in a one or few samples.

Tardigrade abundance was higher in pure host samples than in mixed host samples (Table 1). Mixed host samples had the highest OTU richnesses. Pure host samples of *Pleurozium schreberi* had the highest abundances and diversity indices whilst *Chiloscyphus* had the lowest in all three cases (respectively: Shapiro Wilks $p \leq 0.001$, Kruskal Wallis $df = 5$, $X^2 = 28.315$, $p < 0.001$;

Shapiro Wilks $p = 0.011$, Kruskal Wallis $df = 5$ $X^2 = 25.428$, $p < 0.001$; Shapiro Wilks $p = 0.848$, ANOVA $F_{5,44} = 15.743$, $p < 0.001$; Table 1). The other hosts had intermediate levels of these descriptors.

The sample-based rarefaction curves for 25 mixed host and 25 pure host samples did not reach asymptotes of OTU accumulation, not even when extrapolated to 50 samples in each case (Figure 4). The complete overlap of 95% confidence intervals for the rarefaction curves indicate that no significant differences in OTU accumulation exist between the mixed host and pure host samples.

All host pairings had significantly different tardigrade compositions (PERMANOVA, $p = 0.001$ to 0.049), except between *Leptodontium* and *Zygodon* ($p = 0.123$; Figure 5 and Table 2). The dispersion of *Zygodon* samples in the analysis was much greater than that of the other samples (PERMDISP $p = 0.011$); and the other samples were not significantly different. For interest, the similarity in distributions of OTUs across samples is depicted in Supplementary Material (Figure SM1).

There was no significant relationship between physical distance and tardigrade composition in the mixed host samples (RMA regression $R^2 = 0.006$; $p = 0.098$; Figure 6).

Discussion

Tardigrade abundance and species richness varied considerably between the samples, a pattern that has been shown in the relatively few other studies that have sampled tardigrades quantitatively [20,45,46,65]. In general, bryophyte samples of tardigrades are known to vary in the number of individuals and species richness [49,66]. However, it is difficult to compare tardigrade diversity across different studies where sampling has not been standardised, or even properly described. It would be useful for studies collecting quantitative data on tardigrade composition to describe their methods in detail. Furthermore, despite the practical difficulties in standardising samples of complex, three-dimensional host organisms, we propose that sampling should aim to collect consistent volumes of uncompressed host material. In our study, a standardised sample for bryophytes (mosses, hepatics and liverworts) and lichens of the equivalent of a sphere approximately 4 cm diameter, which represents approximately 4 cm^3 in volume, provided sufficient sampling effort to identify differences between sample groups, but without overwhelming processing effort in the laboratory. Young et al. [43] used a similar sample size to compare successfully the composition and diversity of tardigrade, rotifer and nematode communities in Douglas-fir tree canopies in California.

Table 1. Descriptors of tardigrade communities in host samples: N = total number of tardigrades, Overall S = total number of OTUs in all samples, S = mean ± sd number of OTUs, and H' = mean ± sd Shannon Index based on OTUs. Means sharing a letter within a column were not significantly different.

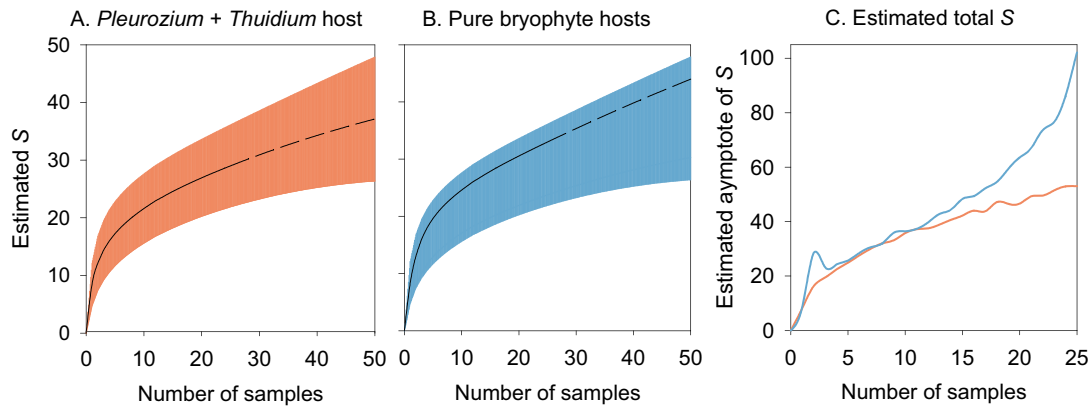| Host	Sample n	N	Overall S	S	H'
Pleurozium + *Thuidium*	25	25.9^{bc} ± 15.9	33	8.9^b ± 3.1	1.9^{ab} ± 0.3
Thuidium	5	30.6^b ± 5.2	18	9.0^b ± 2.9	1.8^{ab} ± 0.4
Pleurozium	5	56.0^a ± 10.7	32	15.4^a ± 1.7	2.2^a ± 0.2
Leptodontium	5	39.2^b ± 22.2	22	10.4^b ± 4.3	1.9^{ab} ± 0.3
Zygodon	5	9.6^{cd} ± 3.0	20	6.0^b ± 2.0	1.6^b ± 0.3
Chiloscyphus	5	3.0^d ± 1.9	5	2.0^c ± 0.7	0.6^c ± 0.4
Overall	50	26.8 ± 19.2	51	8.7 ± 4.2	1.7 ± 0.5

Figure 4. Species accumulation curves for tardigrades species richness (*S*) on the floor of a *Polylepis* woodland in the north of Ecuador: (A) a mixed substrate of *Pleurozium* and *Thuidium* (*n* = 25); and (B) five samples each from pure substrates of five different bryophyte species (total *n* = 25). The continuous line represents the sample-based rarefaction curve for the data set (25 samples), while the dashed line represents the predicted rarefaction curve for up to 50 samples. The shaded areas are bounded by the upper and lower 95% confidence limits for the estimates. (C) Estimates of the species richness asymptote for mixed *Pleurozium* and *Thuidium* samples (orange) and pure bryophyte hosts (blue), using the Chao2 estimator.
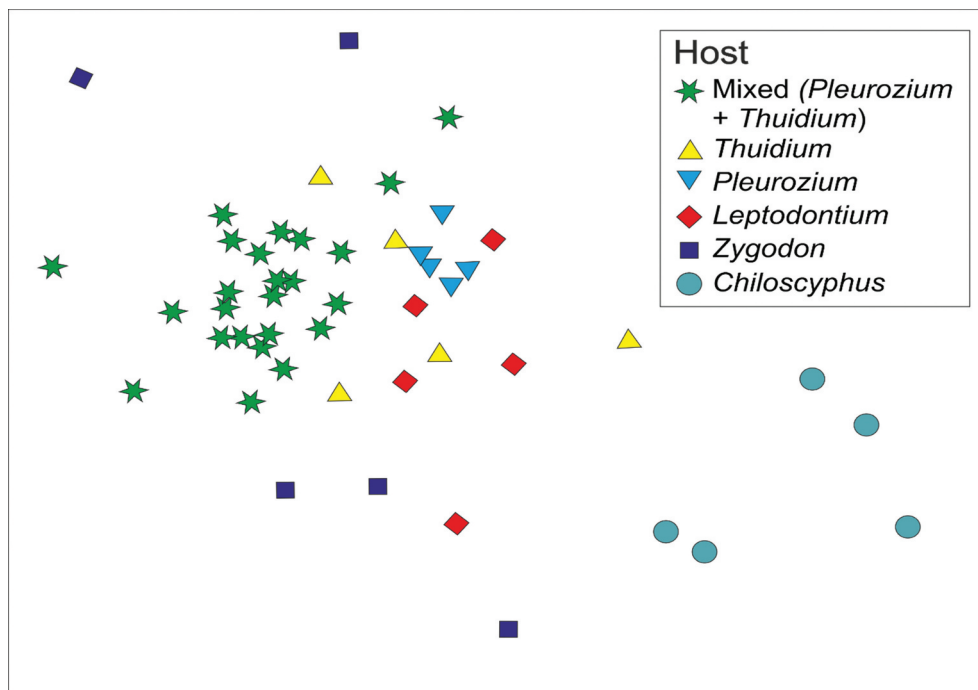

Figure 5. MDS ordination of host samples, based on tardigrade OTU composition, for mixed (*Pleurozium* + *Thuidium*) and pure hosts. Samples located close together in the figure had similar compositions of tardigrades, whereas those further apart were more different in composition.

Table 2. Similarity in tardigrade OTU composition within and between sample types. Diagonals in bold text represent percentage similarity within host samples. The values below the diagonal represent percentage similarity between pairs of host samples. The *p*-values, above the diagonal, show the significance of pairwise Permutational MANOVA tests.

Host	Pleurozium + Thuidium	Thuidium	Pleurozium	Leptodontium	Zygodon	Chiloscyphus
Pleurozium + Thuidium	**49.6**	0.001	0.001	0.001	0.001	0.001
Thuidium	35.7	**46.4**	0.008	0.039	0.049	0.008
Pleurozium	37.5	46.5	**59.5**	0.017	0.007	0.012
Leptodontium	32.6	41.2	48.3	**49.6**	0.123	0.012
Zygodon	24.2	24.0	25.9	27.1	**17.9**	0.009
Chiloscyphus	5.7	12.4	6.3	6.4	5.8	**50.5**

In our samples, eutardigrades were high in OTU richness while heterotardigrades presented low richness. This matches patterns found in quantitative studies of tardigrades in central Spain [20,28]. Eutardigrade diversity is often highest in humid environments, while heterotardigrades are most
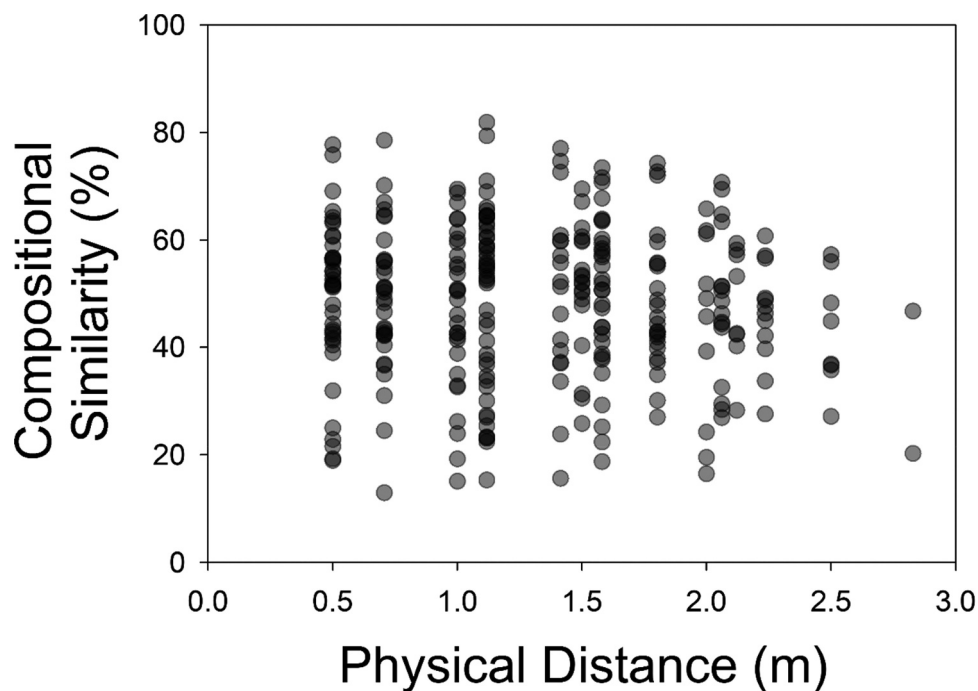
Figure 6. Relationship between physical distance between pairs of mixed host (*Pleurozium* + *Thuidium*) samples and their similarity of tardigrade composition.

diverse in drier conditions [20,41,67,68]. In some previous quantitative studies of tardigrades, heterotardigrades have been found to be more abundant than eutardigrades e.g. [20,28,48],, though the relative abundances of these Classes vary considerably [33,47,49,66]. In contrast, our samples from *Polylepis* forest had more individuals belonging to the Eutardigrada than the Heterotardigrada. *Polylepis* forests in Ecuador are very humid environments [69], where a higher overall abundance of individuals of Eutardigrada might be favoured, given the higher taxon richness of this class in humid habitats more generally.

Macrobiotus species were abundant in most samples, and this genus is one of the most common residents of bryophytes worldwide [39,47,70]. Other tardigrades with a global distribution were also common in our samples, such as *Diphascon, Hypsibius* and *Paramacrobiotus* [71]. Interestingly, several OTUs of *Bertolanius* were present in the samples. This genus has been considered a Holarctic genus [72], but this study extends the presence of the genus into the equatorial mountains of South America. Apart from the biogeographical patterns of genera, it is difficult to compare the tardigrade composition of *Polylepis* forest in more detail because there are so few studies of tardigrade assemblages.

Some tardigrade taxa in our forest samples were sparse, in that they occurred in very low numbers (*e.g. Adropion* cf. *greveni, Adropion* cf. *tricuspidatum, Diphascon arduifrons, Echiniscus bigranulatus*). Many other reports of tardigrade sampling have found sparse taxa [73]. In general, there are several different

forms of sparsity [74], and therefore several different potential explanations for the low abundance and occupancy of taxa in our samples. The potential explanations include fluctuating resources limiting tardigrade numbers, poor resources offered by the host, and the rarity of specific microenvironmental conditions and habitats [74]. Tardigrade numbers can also be reduced by disease, parasitism, predation (sometimes by other tardigrades [75]), and interactions with other meiofauna, including tardigrades [76]. Furthermore, although cryptobiosis helps tardigrades to survive adverse conditions, it is energetically costly and is known to limit reproduction [77,78].

Some taxa were clearly associated more with some hosts than others. The physical structure and chemical composition of particular hosts might determine the abundance of tardigrades. Tardigrades were more abundant and diverse in mosses from the *Polylepis* woodland floor than in the liverwort. Mosses are more structurally complex than liverworts, growing vertically or horizontally, and forming mats or cushions [79]. Thus, the more complex three-dimensional structures of the mosses in our study might provide conditions for a wider number, and potentially a greater diversity, of tardigrades than the simpler structures of the liverwort, *Chiloscyphus* – in a similar way to that suggested for terrestrial and freshwater invertebrates. Suzuki [78] also found that some tardigrades were favoured by the intricate structure of mosses.

However, the relationship between structural complexity of the host and the abundance and diversity of tardigrades is not a simple one. In our study, *Pleurozium* had the highest abundance and diversity,

and whilst the structurally simple *Chiloscyphus* had the lowest, other hosts were intermediate (including the combined samples of *Pleurozium* and *Thuidium*). *Zygodon* had the lowest tardigrade abundance and diversity of the mosses in this study, but the samples varied in the tardigrade taxa that were present (though drawn from a similar pool to that of *Pleurozium* and *Thuidium*). Host structural complexity occurs at different scales, with distinct structural elements, and the interaction of these structural characteristics is likely be more important than any one feature alone.

Although pure *Thuidium* samples had similar numbers and diversity of tardigrades compared with the mixed *Pleurozium* and *Thuidium* samples, pure *Pleurozium* samples had significantly higher tardigrade numbers and diversity. This is contrary to the expectation that more abundant and diverse communities should be found within more diverse habitats [e.g. 80]. If a strong relationship exists between the amount of *Pleurozium* in a sample and the abundance of tardigrades, then the presence of *Thuidium* in the standardized mixed host samples might dilute *Pleurozium's* influence on the abundance and diversity of tardigrades. Even at better-studied scales, where habitat diversity can complement species diversity and influence multifunctionality in ecosystems, the relationship is complex and further consideration of habitat and species diversity together is needed [81]. This seems true for tardigrade-host relationships too.

The hosts provide different structural and micro-environmental habitats for tardigrades (see Figure 2). Although *Pleurozium* and *Thuidium* have a similar pleurocarpus form, *Thuidium* has much smaller leaves arranged tightly around the stem. *Zygodon* and *Leptodontium* appear structurally similar at a coarse scale, but *Zygodon* has dense fine hairs (rhizoids) covering the stem. In a study of a Swedish spruce forest floor, Jönsson [36] found *Pleurozium schreberi* had only intermediate levels of tardigrade abundance and species richness, with two other pleurocarpous bryophytes having the highest levels; the lowest levels were associated with cushions of *Polytrichum formosum* (with an acrocarpous habitat similar to *Zygodon* in our study). It is not clear to what extent the structural characteristics of hosts affect the abundance and diversity of tardigrades within them, but further exploration of this aspect would be worthwhile.

Chiloscyphus, along with other liverworts, has oil bodies within the leaves that might represent a form of chemical defence against herbivory [82,83]. *Chiloscyphus* had the lowest tardigrade abundance and diversity in our study. Certain bryophytes also deter herbivores with phenolic compounds [84]. Among the mosses sampled in this study, *Pleurozium schreberi* has a reportedly higher content of phenolic compounds than *Thuidium delicatulum* [83], but we found *Pleurozium* had the highest tardigrade

abundance and diversity, across a wide range of taxa. This suggests that phenolic content is not the only factor influencing tardigrade occupancy.

Only a few studies have looked for an association between tardigrades and their hosts but the results have been mixed. Bertolani's [85] study found that hosts were not important, whilst other studies have reported that particular tardigrades were linked to specific hosts [86–88]. Drawing conclusions from these studies is difficult because of the great variability in occupancy from sample to sample: often it is not clear from low sampling effort whether these animals show real preferences between hosts or just stochastic differences in sampled occupancy.

We found more microbivore OTUs than any other feeding group, with omnivore and herbivores being found in almost equal numbers. Only one strictly carnivorous tardigrade taxon was present in our samples, but did not impact on the number of herbivores. However, the presence of only one strict carnivore but thirteen omnivores suggests that the ability to utilise a varied diet, including plants, might be favoured in the *Polylepis* forest. Guil and Sanchez-Moreno [28] is the only other study to date to consider trophic groups in natural tardigrade assemblages, but was limited by a relatively small number of samples from leaf litter in central Spain and categorised tardigrades into three feeding groups. In most of these samples, carnivores (+ omnivores) were the most species rich trophic group, followed by herbivores, whilst microbivores were the least species rich. Given that the coarse- and fine-scale habitats were quite different, it is not surprising that our results contrast markedly with their study. More attention to tardigrade feeding groups would be useful to build an understanding of the biotic and abiotic factors that drive their relative abundances.

An important finding of this study was the very high sample effort that was required to estimate tardigrade OTU richness: more than 50 samples would apparently be needed to do this with confidence. Comparing sites and studies only makes sense if an appropriate threshold for effective sampling is met. It is not clear whether the requirement of more than 50 samples suggested by our study is typical of that needed to sample tardigrades in other habitats. This is such a fundamental issue that similar studies in other habitats are urgently required, as part of a wider effort to find effective ways to estimate tardigrade diversity at different scales that is accurate, practical and feasible [46]. Furthermore, for taxonomic studies, greater sampling effort would be more likely to provide the number of individuals needed for the description of new species. Based on a detailed study of several tardigrade species, Stec et al. [89] found that 6–40 individuals of each species were required to adequately estimate mean morphological measurements of characteristic anatomical

structures. Several species in our study did not reach these numbers, even with 50 samples.

In recent years, much effort has been dedicated to analysing patterns of biodiversity for microscopic organisms through the analysis of distance-decay relationships, taxon-area relationships, and local: global taxon richness ratios. Despite this attention, patterns of micro-organism diversity at continental and global scales are still unclear [2]. Studies at finer scales can complement those broader studies [16]. In our samples from widely distributed bryophytes, OTU assemblages were not driven by physical distance over small scales, and did not show spatially predictable patterns at this scale. Thus, it seems that fine-scale differences in environmental conditions (including the distribution of host bryophytes) is much more important in determining tardigrade composition than distance. In other words, the composition of tardigrades in a forest can vary as much between neighbouring bryophytes as between more distant ones.

Conclusions

This work adds to a small number of comparable quantitative studies of tardigrade assemblages at fine scales [20,45,46,65]. The sparsity of some taxa and the variability in numbers from sample to sample, suggest that caution is required in interpreting results from studies which rely on a handful of samples from a locality. Using samples standardised to approximately 4 cm³, our study clearly showed that more than 50 samples are required to estimate tardigrade diversity effectively in *Polylepis* forest. We therefore propose that future quantitative studies should standardize their sample sizes and use appropriate levels of replication to capture local tardigrade biodiversity (and report in detail the precise sampling strategy used). More studies are required to show whether our requirement of more than 50 samples is typical of other habitats. Some tardigrades were restricted to certain hosts, and so collecting from a range of different hosts is also recommended in order to obtain a representative picture of tardigrade diversity.

Disclosure of potential conflicts of interest

No potential conflict of interest was reported by the author(s).

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