RESEARCH LETTER

Application of species richness estimators for the assessment of fungal diversity

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Abstract

Species richness and distribution patterns of wood-inhabiting fungi and mycetozoans (slime moulds) were investigated in the canopy of a Central European temperate mixed deciduous forest. Species richness was described with diversity indices and species-accumulation curves. Nonmetrical multidimensional scaling was used to assess fungal species composition on different tree species. Different species richness estimators were used to extrapolate species richness beyond our own data. The reliability of the abundance-based coverage estimator, Chao, Jackknife and other estimators of species richness was evaluated for mycological surveys. While the species-accumulation curve of mycetozoans came close to saturation, that of wood-inhabiting fungi was continuously rising. The Chao 2 richness estimator was considered most appropriate to predict the number of species at the investigation site if sampling were continued. Gray's predictor of species richness should be used if statements of the number of species in larger areas are required. Multivariate analysis revealed the importance of different tree species for the conservation and maintenance of fungal diversity within forests, because each tree species possessed a characteristic fungal community. The described mathematical approaches of estimating species richness possess great potential to address fungal diversity on a regional, national, and global scale.

Introduction

Fungi are key players of forest ecosystems. They are important for carbon sequestration during wood and leaf decomposition and create essential habitats for microorganisms, protists, fungi and animals (Frankland, 1998). Moreover, mycelia of wood-inhabiting fungi are an abundant nutrition source for saproxylic (wood-inhabiting) invertebrates, and insect taxa are highly dependent on fruit bodies for breeding and feeding (Wheeler, 1984; Fossli & Andersen, 1998).

Attached dead wood in living tree crowns is a natural, omnipresent and essential component of forests (Boddy & Rayner, 1983; Butin & Kowalski, 1986). Nordén *et al.* (2004a) studied the amount and structure of coarse and fine woody debris, attached dead wood, and stumps in temperate woodlands. They concluded that the contribution of organisms, which preferentially live on attached dead wood, is more important for biodiversity than previously thought and needs further investigation (Nordén *et al.*, 2004b). In the topmost and outermost canopy layers, trees are entirely composed of branches and twigs with diameters below 5 cm. In this biologically active interface of earth's terrestrial biomass and the atmosphere, key ecosystem processes are taking place (Ozanne *et al.*, 2003; Körner *et al.*, 2005) and a large part of earth's biodiversity and many organismal interactions still await discovery (Pennisi, 2005; Yanoviak *et al.*, 2005).

Despite these indications, forest canopies are still a little investigated habitat for wood-inhabiting fungi and fungus-like organisms. In the recent edition of *Forest Canopies*, the current benchmark text of canopy research (Lowman & Rinker, 2004), decomposition activities in the forest roof were described only briefly (Fonte & Schowalter, 2004). Moreover, in the comprehensive volume *Biodiversity of Fungi – Inventory and Monitoring Methods* (Mueller *et al.*, 2004), the progression of canopy access techniques (Mitchell *et al.*, 2002) was almost completely ignored (Callan & Carris, 2004).

Research on biodiversity and conservation is often hampered by limited time and financial and personnel resources. Therefore, the description of species abundance distributions followed by an extrapolation of species richness are essential for studies of organismical diversity (e.g. Raaijmakers, 1987; Colwell & Coddington, 1994; Ulrich, 1999; Thompson *et al.*, 2003; Ugland *et al.*, 2003). Numerous mathematical and statistical models were established to describe the species abundance distribution of a given area (for a comprehensive overview, see Magurran, 2004). Many ecologists dealing with macroorganisms are familiar with approaches of extrapolating species richness and assessing the biodiversity of an area beyond data sets. Because of problematic species concepts and definition of individuals, the nonlinearity of fungal development (e.g. Rayner, 1996), or different sampling techniques and measurements, microbiologists and mycologists still have reservations for using these tools. However, the thorough explanation and comparison of different estimators of species richness (e.g. Colwell & Coddington, 1994; Rosenzweig, 1995; Magurran, 2004), the availability of free application for computation of species-accumulation curves and richness estimators (Ugland *et al.*, 2003; Colwell, 2006), and the use of molecular tools that lead to rapidly increasing numbers of fungi and microogranisms has encouraged scientists to use species-accumulation curves and richness estimators to describe microbiological and mycological communities (Tofts & Orton, 1998; Hughes *et al.*, 2001; Bohannan & Hughes, 2003; Unterseher *et al.*, 2005; Lindner *et al.*, 2006; Schnittler *et al.*, 2006).

The aim of the present study is to establish a methodological and theoretical framework for the application of species richness estimators in fungal biodiversity research and to combine information of alpha diversity with ecological patterns of the organisms (e.g. spatial distribution and substrate specificity). Moreover, one hypothesis – nonparametric methods of species richness estimation (e.g. the Chao 2 estimator) work especially well for microbial biodiversity data (Bohannan & Hughes, 2003) – was tested for own data.

Materials and methods

The work is based on the data obtained during fieldwork from 2002–2005. Using a construction tower crane installed for observation purposes in a temperate mixed deciduous forest in Leipzig, Central Germany (Morawetz & Horchler, 2004), dead branches of living trees were removed from the forest canopy. Sampling height was between 10 and 33 m above the ground. The samples were checked for the occurrence of wood-inhabiting fungi, and mycetozoans on decorticated twigs (Unterseher *et al.*, 2005, 2006; Schnittler *et al.*, 2006). The identification was achieved using morphological characters of fruit bodies and sporocarps. Details of canopy access, sampling design, and species lists are described elsewhere (Unterseher *et al.*, 2005; Schnittler *et al.*, 2006).

Application of diversity indices and species richness estimators

Sample-based data were used for the calculation of diversity indices and estimators of species richness with EstimateS Version 8 (Colwell, 2006). Fisher's diversity index, alpha (α, Fisher *et al.*, 1943), and the Q statistic (Q, Kempton & Taylor, 1978) were used to describe species richness.

Six estimators of species richness were compared: Chao 2 richness estimator (Chao, 1987), incidence- and abundance-based coverage estimator [incidence-based coverage estimator (ICE) and abundance-based coverage estimator (ACE), Chao *et al.*, 2000], first-order Jackknife richness estimator (Jackknife 1, Burnham & Overton, 1979), Bootstrap richness estimator (Smith & Belle, 1984), and Michaelis–Menten richness estimator (Raaijmakers, 1987). In contrast to Chao 1, which uses the number of singletons and doubletons (species represented by exactly one and two individuals) and thus an abundance-based estimator, Chao 2 is an incidence-based estimator of species richness, which relies on the number of unique units and duplicates (species found in only one and two sample units (Chazdon *et al.*, 1998).

A further richness estimator was used for our studies. This method of extrapolating species richness was recently introduced by Gray and is described at length in Ugland *et al.* (2003). The first step is to calculate average species-accumulation curves for several subplots that is the average species richness when one, two, three, etc. subplots are randomly drawn from the entire data set. As subplots we have chosen the four tree species *Acer pseudoplatanus* (8 individual trees), *Fraxinus excelsior* (10), *Quercus robur* (8), and *Tilia cordata* (10). To estimate the total species richness on more than four tree species, the upward shift of the curve, which resulted by connecting the four terminal points of each of the mean accumulation curves, was described mathematically. After semi-log transformation of the terminal points of each of the subplot curves, a linear model was fitted to the data using the linear model function of the software package R (R Development Core Team, 2005).

To predict the species richness of larger areas with Gray's method, a random sample was drawn from the main unit consisting of five subplots, each containing 20 randomly drawn samples. By this means, the estimator became independent of tree species and dependent only on the number of samples per area. Within the crane site (1.6 ha), 128 samples were examined for the occurrence of fungal organisms. All predictions of species richness of larger areas were therefore based on the assumption that research would continue with a comparable sampling effort, in this case 80 samples per hectare.

To evaluate the reliability of species richness estimators for canopy fungi, a second series of the same analyses was conducted. Data from a nearly complete survey of mycetozoans in the same habitat were used (Schnittler *et al.*, 2006). Despite the comparatively small data set it was considered appropriate for this purpose, because between 82% and 88% of the expected species have been recovered and the resulting rarefaction species-accumulation curve has almost reached an asymptote (Schnittler *et al.*, 2006).

Communities of wood-inhabiting fungi in different habitats

To advance our myco-ecological understanding of forests, we combined the analysis of fungal species richness with an analysis of fungal species composition. Preferences for the tree species *A. pseudoplatanus, F. excelsior, Q. robur, and T. cordata* were assessed with nonmetric multidimensional scaling (NMS or NMDS), a frequently used method to analyse multidimensional data (for cryptogams see e.g. McCune *et al.*, 2000; Schnittler *et al.* 2006). The program PC-ORD for Windows Version 5.0 (McCune & Mefford, 2006) was used with the 'slow and thorough' autopilot option (maximum number of iterations = 500, instability criterion = 1 E-8, starting number of axes = 6, number of real runs = 250, number of randomized runs = 250) and the Sorensen (Bray–Curtis) distance measure. Before NMS, species present in less than five samples were deleted from the sample–species matrix to reduce noise from the ordination. It was followed by a 'Beals smoothing' transformation to reduce the number of zeros in the data matrix (Ewald 2002; McCune & Grace 2002).

Results

Estimated species richness

The numbers of observed species were 146 for all data (wood-inhabiting fungi and mycetozoans) and 37 for mycetozoans (Fig. 1; Table 1). The estimated species richness for all data ($\pm$ 1 SD) was 208 (ACE), 210 (ICE), 200 $\pm$ 19 (Chao 2), 171 (Bootstrap), 201 $\pm$ 8 (Jackknife 1), and 178 (Michaelis–Menten) (Fig. 1a). The estimated species richness for mycetozoans ($\pm$ 1 SD) was 44 (ACE), 44 (ICE), 41 $\pm$ 4 (Chao 2), 41 (Bootstrap), 45 $\pm$ 3 (Jackknife 1), and 41 (Michaelis–Menten) (Fig. 1b). The application of Gray's method with the four tree species as subplots is shown in Fig. 2. Calculation of species richness for parts of the investigation site (i.e. four tree species, 0.8 and 1 ha), and extrapolation of species richness to both a larger number of tree species (10 and 15), and the whole nature conservation area (270 ha) is shown in Table 2. Gray's method of extrapolating species richness was also applied to the data of mycetozoans (Table 2).

Diversity of wood-inhabiting fungi on different tree species

The NMS of wood-inhabiting fungal communities on different tree species resulted in a distinct grouping of fungi and samples in ordination space (Fig. 3). The majority of species is arranged in four clusters and superimposed with four sample groups. The sample groups are concordant with the four tree species on which the fungi occurred. Further samples were predominantly colonized by ubiquitous fungi and are therefore located remote from the sample clusters of the corresponding tree species, as are also the ubiquitous fungi. Additionally, the species-accumulation curves and the Chao 2 richness estimators are shown for the tree species and all data. The observed species richness was 23 (*A. pseudoplatanus*), 17 (*F. excelsior*), 32 (*Q. robur*), and 48 (*T. cordata*). The species-accumulation curves continued to rise linearly; a saturation of species richness was not observed. The Chao 2 richness estimators behaved differently. For *Tilia* (89 predicted species), *Acer* (44), and *Fraxinus* (23), the number of estimated species richness declined at the end; for *Quercus* (104) and all data (200) there was no end in sight.

Discussion

Definition of the fungal individual

For this study, statistical tools were applied, which are traditionally used by ecologists dealing with macroorganisms and discrete entities, namely individuals. Because filamentous fungi, protists, and other microorganisms often lack characters for a secure definition of individuals, some concerns about the fungal individual seem appropriate. Previous studies revealed nonlinear (chaotic) patterns of fungal growth (Rayner, 1996) and complex spatio-temporal organization of fungal communities on dead wood (Boddy & Rayner, 1983; Chapela & Boddy, 1988). Numerous individuals of the same species were observed on the same branches, each producing its own stroma or fruit body. Other fungi with expanded mycelia produced several fruit bodies per individual. During our studies, the cryptic nature of fungi and mycetozoans (mycelia of wood-inhabiting fungi, aphaneroplasmodia of myxomycetes) usually made the delimitation of individuals impossible. The numbers of individuals per species and per sample are therefore rough estimates based upon the number of fruit bodies. These estimates again are based upon the literature (e.g. Boddy & Rayner, 1983; Boddy, 1992; Heilmann-Clausen & Boddy, 2005), the communication with experts, and own experience. The analysis however was not affected by the uncertain extent of fungal individuals. Plotting the curves as a function of the number of samples instead of the number of individuals did not change the outcome of estimators (not shown).

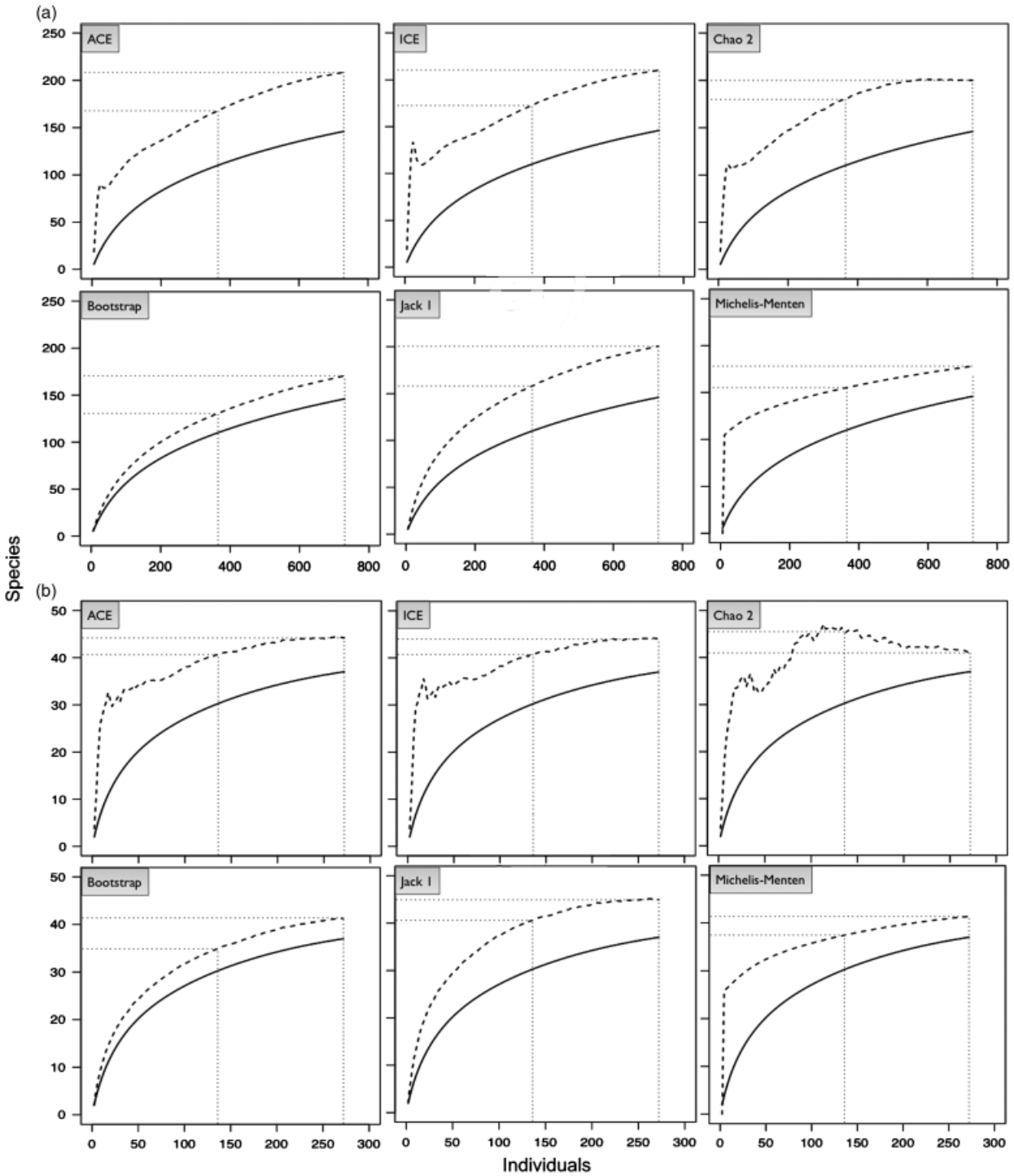

Fig. 1. Estimators of species richness (dashed lines) and the rarefaction-species-accumulation curve (solid lines) for all data (a), and for mycetozoans (b). ACE, Abundance-based coverage estimator; ICE, Incidence-based coverage estimator; Jack 1, first-order Jackknife richness estimator. The dotted lines indicate 50% and 100% sampling effort.

Table 1. Indices of species richness

				Diversity indices	
	Species	Individuals	Samples	α	Q
All data	146	730	128	54.88	50.78
Mycetozoans	37	272	128	11.56	11.97
Wood-inhabiting fungi	109	458	128	45.25	41.63
Acer pseudoplatanus	60	367	44	20.37	20.19
Fraxinus excelsior	53	212	67	22.68	22.99
Quercus robur	67	242	50	30.66	25.47
Tilia cordata	67	176	62	39.48	38.23

The observed number of species, of individuals, the number of samples, and the diversity indices Fisher's alpha (α) and the Q-statistic (Q) are shown for all data, for *Myxomycetes* and *Myxomycete*-like organisms (*Mycetozoans*), for wood-inhabiting fungi, and for each of the four observed tree species.

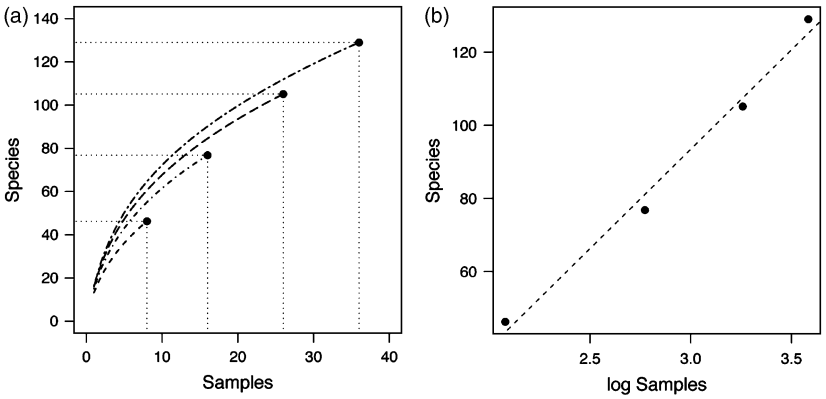

Fig. 2. Results of Gray's method of extrapolating species richness (Ugland *et al*., 2003). (a) Mean species-accumulation curves of four subplots. The lower curve in Fig. 2a (- - -) resulted from calculating the means of all four combinations to draw one single subplot at random (*Acer pseudoplatanus* or *Fraxinus excelsior* or *Quercus robur* or *Tilia cordata*). The next curve resulted by averaging all six combinations of two subplots and so on. Finally the topmost curve resulted from the only combination of drawing all subplots (*A. pseudoplatanus* and *F. excelsior* and *Q. robur* and *T. cordata*). (b) Semi-logarithmic transformation of the terminal points from (a). The regression line (- - -) follow the equation $y = 54.34 \log(x) - 69.55$ (adjusted $R^2 = 0.98$) which was used then to extrapolate species richness.

Analysis of species richness

Because of the large number of rare species in our data, all species-accumulation curves except that for mycetozoans continued to rise almost linearly. For the latter organisms, rare species were lower compared with wood-inhabiting fungi. The diversity indices α and Q also showed this relation (Table 1). We therefore consider the use of the indices in combination with sample-based rarefaction species-accumulation curves appropriate to describe the species richness of fungal organisms at the investigated forest stand.

The Chao 2 richness estimator performed best with our mycological data

A great advantage of the Chao 2 richness estimator for mycological studies is that it does not require precise information on the number of individuals per sample. Chao 2 is an incidence-based estimator of species richness, which relies on the number of unique units and duplicates (species found in only one and two sample units) and not on the number of singletons and doubletons (species present with one and two individuals).

For mycetozoans, it was evident that Chao 2 reached its final value with the lowest sampling effort (41 species with 30% sampling effort; Fig. 1b). Although an overestimation of species richness occurred afterwards, a stable asymptote of 42 ± 1 species was reached after 68% sampling effort. ACE performed equal to ICE and also approached an asymptote before sampling effort reached 100%. Both estimators needed larger sample sizes to calculate the final values of 44 species. Comparable results were obtained during other studies of both macro and microorganisms with unequal catchability (Chao, 1987; Colwell & Coddington, 1994; Hughes *et al*., 2001; Bohannan & Hughes, 2003; Magurran, 2004). Bootstrap and Michelis–Menten estimators performed poorly even for the almost complete survey of mycetozoans (Fig. 1b). From our mycological data we cannot recommend them as reliable measures of species

Table 2. Gray's method of estimating species richness (Ugland *et al.*, 2003) for parts of the investigation site (4 tree species, 0.8 and 1 ha), for the crane site itself, for a larger number of tree species (10 and 15), and for the whole nature reserve

	'Area' of interest	Species observed	Gray's method (1 SD)
All data	4 tree species	129	125 (4)
	10 tree species	–	175 (7)
	15 tree species	–	197 (9)
	50% sampled (0.8 ha or 64 samples)	109	101 (2)
	1 ha	121	112 (2)
	crane site (1.6 ha)	146	135 (3)
	Nature reserve (270 ha)	–	392 (2)
Mycetozoans	4 tree species	36	35
	10 tree species	–	46
	15 tree species	–	51
	50% sampled (0.8 ha or 64 samples)	30	30
	1 ha	33	33
	Crane site (1.6 ha)	37	38
	Nature reserve (270 ha)	–	97 (2)

For a better evaluation of the reliability of Gray's method, the observed species richness is also shown.

richness. It is important to note at this point that the species-rich guild of corticolous mycetozoans was not considered – the bark was removed completely before the incubation of woody pieces in moist chambers (Schnittler *et al.*, 2006) – and that the investigation of new small-scale habitats and niches definitely would affect the outcome.

On the basis of an obviously insufficient sampling effort to capture most of the expected fungal organisms from the forest canopy, Chao 2 was the only estimator that reached a distinct curve flattening at 200 species for all data (Fig. 1a). Comparing the curve progression of Chao 2 for all data with that of the mycetozoans, the final flattening (Fig. 1b) could be interpreted as a slight overestimation of the effective species richness. Note that the curves in Fig. 1a are composites of mycetozoans data that are almost saturated in terms of species richness and fungal data that are clearly unsaturated. The resulting graphs therefore do not show this ambiguity.

How many species can be expected on dead attached branches?

Approximately 220 fungal species (45 mycetozoans and 175 wood-inhabiting fungi) were estimated to occur on dead attached branches in the canopy of the crane site if sampling continued. However, even the best performing estimator requires a certain sampling effort before stable values are calculated for a given habitat or site of interest. The number of samples from individual tree species was obviously insufficient to obtain stable values of fungal species richness for each host (Fig. 3). Consequently, the predicted species richness of 220 did not conflict with the data of Fig. 3 (the Chao 2 estimator for wood-inhabiting fungi alone already reached 200 species). Based upon our data and published studies (e.g. Colwell & Coddington, 1994), Chao 2 overestimates species richness before the predicted species number declines and reaches a stable asymptote.

Taking into account the ratio of *c.* 220 expected to 146 observed fungal species at the crane site and assuming the same ratio for other sites and larger areas, the fungal species richness, if predicted with Gray's method, would rise from 175 to 240 for 10 tree species, from 197 to 270 for 15 tree species, and from 392 to 537 for the whole nature reserve (Table 2).

Despite the pressure to specify concrete species numbers for policy makers and the public, we should be cautious to extrapolate observed species richness in real ecosystems beyond data sets. This is because previously unsampled cryptic niches might be revealed from a more extensive sampling schedule, thus affecting the analysis. As reported above, only morphological information of the fungal species was available for data analysis. Many taxa within our species lists are known or presumed to form cryptic species assemblages (e.g. *Phoma*, corticioid fungi) which can, if at all, be elucidated only by sequence analysis (Boerema, 1997; Larsson, 2007). All estimators presented in the paper can be viewed as truly conservative.

Conclusions and perspectives

Based upon our experience, we recommend Chao 2 to be applied to uncover the number of species that could be found in a particular investigation site with larger sampling effort. If new sites are investigated with comparable sampling effort, Gray's estimator of species richness could provide a reliable and conservative estimator for the expected number of species of larger areas (Ugland *et al.*, 2003). An advantage of Gray's method is that it fits extremely well for data from homogeneous as well as

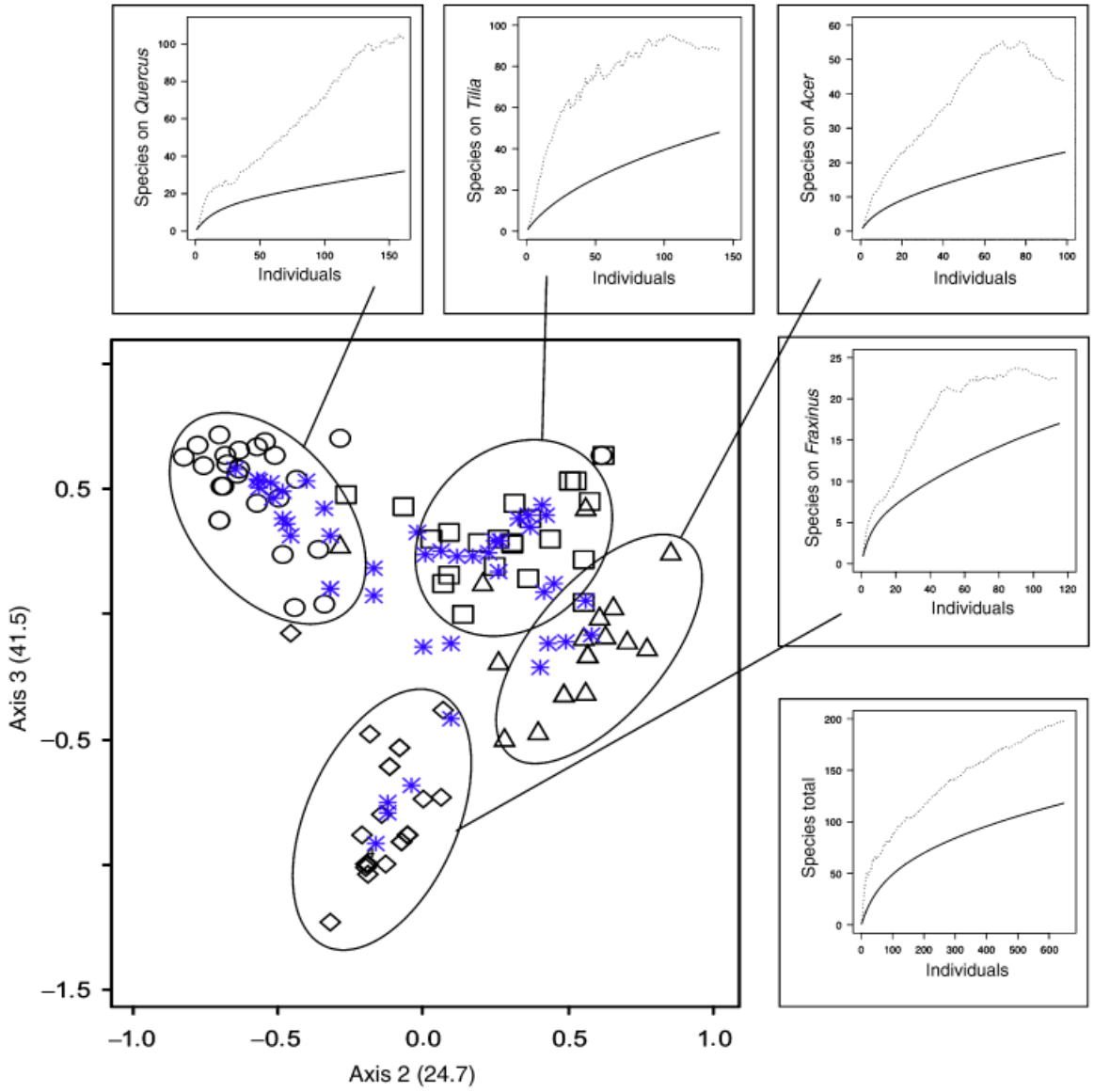

Fig. 3. Ordination biplot of nonmetrical multidimensional scaling with the two most important axes. Values in parentheses are the percentage of total explained variation of species data. The data contained wood-inhabiting fungi from the trees *A. pseudoplatanus, F. excelsior, Q. robur,* and *T. cordata* with five or more individuals per sample. The corresponding rarefaction-species-accumulation curves and the Chao 2 richness estimators are shown above and right to the ordination.

heterogeneous environments. The free availability of the algorithms in an excel spreadsheet (download at http://folk.uio.no/johnsg) makes it easy to use for own data from different environments with different organisms.

It would be of importance to evaluate Chao 2 and Gray's predictor of species richness for the data used by Hawksworth (1991, 2001) and others to estimate the 1.5 M fungal species worldwide. Nearly all extrapolations of fungal diversity are based on a number of assumptions and correction values such as ratios of fungi to their associated hosts as crosssections over large geographic areas (e.g. tropical, temperate, alpine communities), known anamorph–teleomorph relationships, or the consideration of understudied fungal groups and habitats (Hawksworth, 1991, 2001; Fröhlich & Hyde, 1999). Consequently, the extrapolations differ strongly, depending on the taxonomic group, the geographical area under investigation, and the number of specialists involved in the surveys and received sceptical considerations (e.g. May, 1991). The application of mathematical species richness estimators (Burnham & Overton, 1979; Chao, 1987; Raaijmakers, 1987; Palmer, 1990; Chao *et al.*, 2006) could effectively minimize criticism by enhancing consistency of previous and future studies of fungal diversity and by considering genetic diversity (Bohannan &

Hughes, 2003). This last point is crucial for applied biodiversity because it could then keep pace with the progress of molecular phylogeny. As species richness is the elementary basis of biological diversity, tools to assess the number of species are urgently needed to keep pace with the rapid advancements of genetics and phylogeny, which reveal enormous species numbers, especially microorganisms from short surveys (Hawksworth & Colwell, 1992; Tiedje *et al.*, 1999; Hughes *et al.*, 2001; Bohannan & Hughes, 2003; Forney *et al.*, 2004).

The question of ecological studies and biodiversity management is generally not the species number *per se*, but what specific species and communities are present and why. Therefore, the joint analysis of species richness and species composition of guilds or ecological niches is of importance if the biodiversity of certain habitats or sites of interest are compared.

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