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Field sampling methods for investigating forest-floor bryophytes: Microcoenose vs. random sampling

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Abstract: Because of the high importance of bryophytes in forest ecosystems, it is necessary to develop standardized field sampling methodologies. The quadrat method is commonly used for bryophyte diversity and distribution pattern surveys. Quadrat size and the position of quadrats within the studied area have a significant influence on different analyses. The aim of the present study was to define the minimum quadrat size appropriate for sampling ground bryophytes in temperate beech forests, to compare two different field sampling methods for research on ground bryophytes, the random and microcoenose methods; and to test the adequacy of the microcoenose sampling method in temperate beech forests. Research was carried out on Fruška Gora mountain (Serbia) at four different sites. All sites contained temperate broadleaf forest vegetation, predominantly *Fagus sylvatica*, but also included various other tree species. Systematic sampling based on nested quadrats was used to determine the minimum sampling area. Random sampling was performed using 10 or 20 microplots (minimum area quadrat), randomly located within 10x10 m plots. Microcoenose sampling is a systematic sampling method based on the fact that every bryophyte fragment on the forest floor is a separate microcoenose. These methods were compared using the following criteria: species richness; Shannon's diversity index and evenness measure; coverage of dominant species, and the time needed for sampling. The microcoenose sampling method has proven to be highly applicable in temperate beech forests in terms of species richness and diversity, in contrast to random sampling, which was not suitable for bryophyte flora with a patchy distribution.

Key words: bryophytes; microplots; sampling; forests; diversity

INTRODUCTION

Bryophytes (mosses, liverworts and hornworts) play important roles in forest ecosystems by contributing to species richness [1,2] forest biomass [3,4], water regime and nitrogen budget regulation [5,6], while also providing a microhabitat for other organisms [7]. In general, bryophytes are important components of "forest integrity" [8]. Due to this fact, it is necessary to include bryophytes in all forest ecosystem studies. Vegetation and phytosociological studies in Europe use well-developed and standardized methodology [9-12], but in the majority of these studies, bryophytes have been ignored, despite the fact that these plants have a large influence on ecosystems, phytocoenosis and habitat conditions.

A globally accepted method for the quantification of bryophyte abundance in forest communities is still lacking, but there are several approaches that have been standardized. Three commonly used approaches for quantitative bryophyte sampling in forests are: the line intercept method [13-16], floristic habitat sampling [17,18] and the quadrat method [1,19-26]. The main problem with the line intercept method is an increased probability of missing small species [16]. Floristic habitat sampling (FHS) is a method based on the use of microhabitats within the stand as a sampling unit [17]. This method is similar to floristic sampling, and its main advantages are the high possibility of recording rare species and high applicability in bryophyte research over large areas [17]. The main disadvantage of FHS is the fact that this method does not estimate abundance well, so it is not completely appropriate for estimation of statistical inference or good abundance [18]. The quadrat method is commonly used in bryophyte studies of diversity and distribution patterns. There are several disadvantages in using the quadrat

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method for quantifying bryophytes. The first problem concerns the appropriate quadrat size. Using too small a quadrat can lead to the exclusion of some very important species. The minimum area concept for determination of the minimal appropriate quadrat size (based on species area curves) depends on the scale. According to some authors [27,28], it is hard to fix the minimum area that could properly catch a sufficient proportion of total diversity in any type of habitat. In general, species area curves rarely reach complete saturation [29], and species numbers increase with quadrat enlargement; however, at some point this enlargement slows down. Moravec [30] suggested using the criterion of similarity and confirmation of minimum area by stopping the increase of average similarity through enlarging quadrat size. Although determination of quadrat size by species area-curves is not an "ideal" solution, it is the most efficient and most commonly used. The second problem is how to find an appropriate method for quadrat positioning. Is it better to use a completely random approach, or some form of systematic quadrat positioning? The problem with random positioning is that it mainly excludes the existence of different microhabitats within the plot (phytocoenosis) where the study is performed; this problem can be bypassed by systematic sampling [31]. The third problem with the quadrat method is that different authors use different quadrat sizes (microplots), making results from different studies less comparable [28].

Bryophytes show variations in distribution patterns in different types of ecosystems, and therefore, sampling methods for quantification are highly dependent on the type of ecosystem, environmental factors and the aim of the research [7]. Jiang et al. [32] developed a microcoenose sampling method for ground bryophyte flora in different types of forest vegetation in China, which provides sufficient information in terms of species richness and distribution of bryophytes. However, it is not known if this sampling method is applicable or advantageous in temperate forests in comparison to random sampling methods.

The aim of the present study was to address the following issues: (i) what is the minimum quadrat size for the quantification of ground bryophyte flora in temperate forests dominated by *Fagus sylvatica*, and (ii) which model performs better – completely random sampling or the microcoenose sampling method.

MATERIALS AND METHODS

Study site

This research was performed during March-April 2016 on Mt. Fruška Gora, located in the north of Serbia in the southern part of the Pannonian plain (Fig. S1), between 45°0' - 45°15' N and 16°37' - 18°01' E. This mountain is surrounded by the Danube alluvial plain in the north and east, and by two loess plateaus in the south and west. The highest peak is Crveni Čot (539 m a.s.l.). Geologically it is a very diverse area. The largest part is composed of siliceous rocks, and the vegetation probably dates from the Tertiary, because glaciation did not have a significant impact on this mountain [33]. There is a dense hydrological network composed of groundwater, karst springs, mineral and thermal springs, streams (constant and periodical) and some standing water [34]. There are three types of soil on Fruška Gora: chernozem, brown forest soil and brown calcareous soil [35]. This area lies in a mild-continental central European climatic region [36]. The highest precipitation levels are in May-June, September and October [33]. The lowest average temperature is in January and the highest in July [37]. Due to its natural value, Fruška Gora was declared a National Park in 1960. The majority of the protected area is under forest vegetation.

For this study, four localities on Mt. Fruška Gora were chosen, all under typical forest vegetation: these were Iriški Venac-Stražilovo (IS), a beech (*Fagus sylvatica*) forest dominated by bryophytes in the ground layer; Papratski Do (PD), a mixed forest with *F. sylvatica* as the dominant species and significant participation of *Carpinus betulus, Quercus petraea* and *Tilia platyphyllos*; Vrdnik (V), a mountain beech forest with dominant species *F. sylvatica* and *Q. petraea*, and Dumbovo waterfall (D), a monodominant beech forest with absolute domination by *F. sylvatica*.

Sampling scheme

In the present study, bryophytes were considered *sensu lato* (i.e. including representatives of mosses and liverworts, while hornworts were not found in this area). In addition to species found on the soil, bryophytes that grow on small rocks and roots at elevations less than 5 cm above the soil surface were also considered to be

forest floor bryophytes. The main reason for this is that many species in this area are polyedaphic, and many bryophyte patches are spread across different substrates.

At each locality, a sampling area was chosen in the central part of the selected forest sites. Five plots (10x10 m) were then randomly chosen within the boundaries of these sampling areas. On these plots, all bryophyte taxa were listed to obtain the actual species numbers and calculated as the average species number in five 10x10 m plots. To obtain actual coverage values (%), total coverage of all bryophytes, as well as coverage by dominant bryophyte species (species with the highest abundance on the plots) was measured on 10x10 m plots within each site. Actual species numbers and coverage values were used for comparison of the species number and coverage recorded using different sampling methods and calculated as the proportion on 10x10 m plots.

Bryophytes were identified in the field or in the laboratory, and deposited in a herbarium.

At each study site, a minimum sampling area was determined using a systematic sampling method [32] with some modifications, as follows: five plots (10x10 m) were delineated with nested quadrats with dimensions of 10x10 cm, 20x20 cm, 50x50 cm, 1x1 m and 2x2 m (Fig. S2); the distances between the sampling quadrats was equal; species richness and abundance were measured in all 2x2 m quadrats (125 in total).

The minimum sampling area (microplot) was used for testing two different sampling methods. First, a random sampling method was performed using 10 randomly located microplots (Fig. S3A) within each 10x10 m plot (for a total of 50 microplots per study site). Then, the number of microplots was increased to 20 (Fig. S3B) for each plot (for a total number of 100 microplots per study site) in order to test the appropriate number of minimum area quadrats (microplots) for random sampling. Randomness was achieved by placing a wooden frame (50x50 cm, delineated with 1x1 cm quadrats) within the boundaries of the plot (10x10 m). Microplots without bryophytes were also included in the analysis.

Second, a microcoenose sampling method [32] was employed. In this case, every bryophyte fragment was considered to be a microcoenose. Plots (10x10 m) were delineated on 25 grids (2x2 m). The microplots were thrown in the center of the largest bryophyte fragment in each of 25 grids (Fig. S3C). Grids without bryophytes were included in the analysis.

Data analysis

To determine minimum quadrat size, the following indices were used: species richness (S), the number of species in each analyzed quadrat or plot (10x10 m), and the Sørensen similarity index [38]. A qualitative minimum area curve (species-area curve) was constructed [29] for all studied sites in order to determine the minimum appropriate quadrat size. The turnover point in each species-area curve was determined by the tangent method [39]. A similarity area curve was constructed for confirmation of the species-area curves. The turnover point in each similarity-area curve was based on the point where the similarity values were greater than 80% [40].

For data analysis, the average coverage and species richness of all microplots for each sampling area were used. Four criteria were used for testing the usability of these sampling methods for some quantitative diversity measurements: (i) species richness (S) gained in different types of sampling, (ii) Shannon's diversity index (H') and evenness measure (J') [41], (iii) coverage of dominant species, and (iv) the time needed for sampling, expressed in min. The sampling time was measured only at site D and included species identification, packing of species impossible to identify in the field and measuring of species coverage in all individual microplots. Statistical analyses were performed using the t-test in STATISTICA® ver. 13.2 software [42]. The diversity index was calculated and compared using PAST ver. 3.15 [43].

RESULTS

Actual species number and actual coverage of bryophytes

The total number of species listed by 10x10 m plot size at V, D, IS and PD were 23, 35, 28, 21, respectively. Differences in species richness at the four studied sites were probably related to different ecological conditions in each type of forest. The actual coverage of

bryophytes on the 10x10 m plots was similar for V, D and PD (23%, 35% and 21%, respectively), while it was much higher for the locality IS, where it reached 91%.

Minimum area determination

Based on the qualitative minimum area curve (species-area curve), the turnover point was found to be a quadrat size 50x50 cm (0.25 m^2) for all investigated sites (Fig.1).

Using a similarity area curve (Fig.2) for each site, the turnover point was found to be a quadrat size 0.25 m^2, which is based on the similarity between quadrats

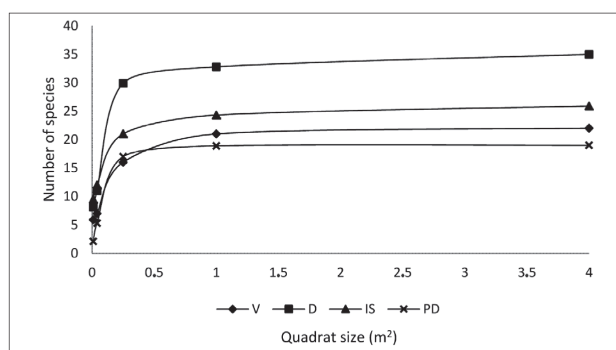

Fig. 1. Species-area curve for each study site based on the method of nested quadrats (V –Vrdnik; D – Dumbovo; IS – Iriški Venac-Stražilovo; PD – Papratski Do).

in which it was higher than 80%. All subsequent quadrats were not significantly different from the 50x50 cm quadrats.

Considering the abovementioned characteristics, a quadrat size of 50x50 cm was selected as the minimum quadrat size (microplot) for testing the random and microcoenose sampling methods.

Species richness

The random sampling method was tested as an appropriate method for reducing subjectivity in field sampling. The main problem with this method was the difference between the species numbers recorded in all microplots (10 or 20 per plot) and the actual number of species at all studied sites. In the first case, based on 10 randomly located microplots (50x50 cm) at all four localities, a statistically significant difference ($p<0.05$) was found between the actual species number and the recorded species number for all 50 microplots (Table 1).

In the second case, based on 20 randomly located microplots, only one study site (IS) was found without a statistically significant difference ($p<0.05$) between the actual species number and measured species number (Table 2).

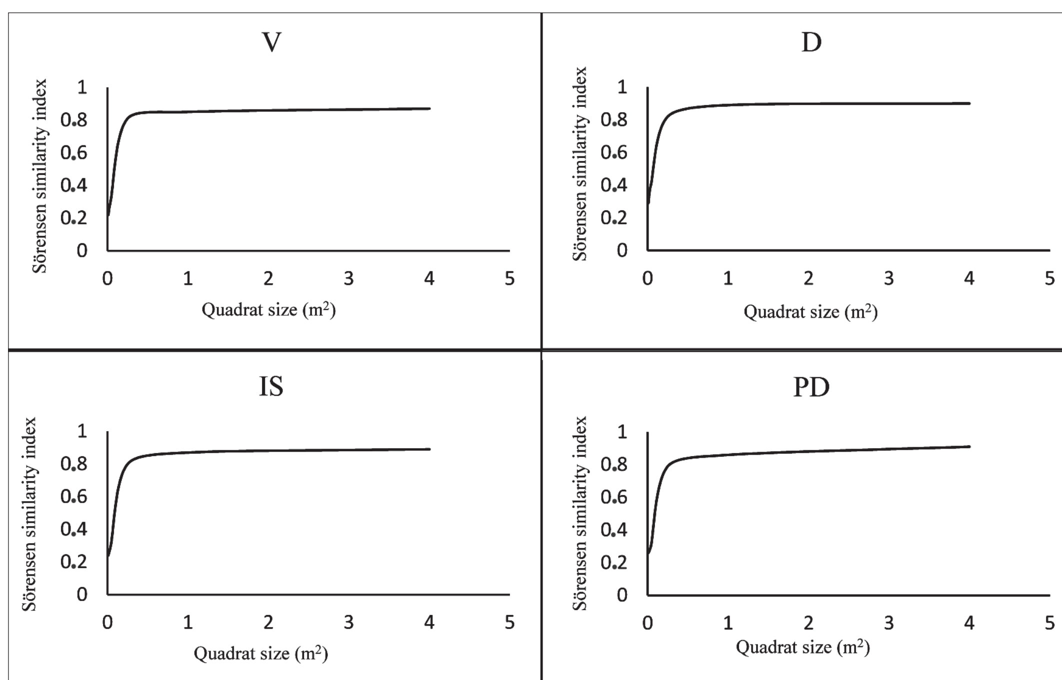

Fig. 2. Similarity area curves for each studied site based on the method of nested quadrats (V – Vrdnik; D – Dumbovo; IS – Iriški Venac-Stražilovo; PD – Papratski Do).

Table 1. Comparison of recorded species number and actual species number for each studied site using the random sampling method with 10 microplots.

Study site[1]	Actual species number[2]	Recorded species number[3]	Number of microplots without bryophytes[4]	p
V	23	9	3	0.0007*
D	35	11	4	0.0013*
IS	28	21	0	0.0076*
PD	21	4	2	0.0003*

[1]V – Vrdnik; D – Dumbovo; IS – Iriški Venac-Stražilovo; PD – Papratski Do
[2]Average species number in all 5 plots (10x10 m); the means are rounded off
[3]Average species number recorded in all 50 microplots (50x50 cm); the means are rounded off
[4]Average number of microplots without bryophytes from all 5 plots (10x10 m)
*statistically significant difference (T-test, independent variables) for $p<0.05$

Table 2. Comparison of recorded species number and actual species number for each studied site using the random sampling method with 20 microplots.

Study site[1]	Actual species number[2]	Recorded species number[3]	Number of microplots without bryophytes[4]	p
V	23	12	7	0.00076*
D	35	11	8	0.00002*
IS	28	28	0	0.06516
PD	21	10	5	0.00049*

[1] V – Vrdnik; D – Dumbovo; IS – Iriški Venac-Stražilovo; PD – Papratski Do
[2]Average species number in all 5 plots (10x10 m); means are rounded off
[3]Average species number recorded in all 100 microplots (50x50 cm), means are rounded off
[4]Average number of microplots without bryophytes from all 5 plots (10x10 m)
*statistically significant difference (T-test, independent variables) for $p<0.05$

Table 3. Comparison of recorded species number and actual species number for each studied site using the microcoenose sampling method

Study site[1]	Actual species number[2]	Recorded species number[3]	Number of microplots without bryophytes[4]	p
V	23	21	8	0.37236
D	35	32	9	0.13596
IS	28	28	0	0.34659
PD	21	20	12	0.10532

[1] V – Vrdnik; D – Dumbovo; IS – Iriški Venac-Stražilovo; PD – Papratski Do
[2]Average species number in all 5 plots (10x10 m); the means are rounded off
[3]Average species number recorded in all 125 microplots (50x50 cm); the means are rounded off
[4]Average number of microplots without bryophytes from all 5 plots (10x10 m)

Table 4. Comparison of coverage of dominant species on all studied sites using three different sampling methods.

Study site[1]	Actual coverage[2]	RS10[3]	p	RS20[4]	p	MS[5]	p
V	12.4	4.8	0.000565*	7.2	0.002303*	11.8	0.64793
D	15.2	6.2	0.029494*	7.6	0.047017*	14.8	0.92835
IS	30.4	14.6	0.000086*	22.4	0.005522*	29.4	0.62712
PD	8.2	3.4	0.016167*	3.8	0.038868*	8	0.91909

[1]V – Vrdnik; D – Dumbovo; IS – Iriški Venac-Stražilovo; PD – Papratski Do
[2]Average coverage (%) of dominant species measured on plots (10x10 m)
[3]Average coverage (%) of dominant species measured by the random sampling method with 10 microplots, transferred to plots (10x10 m)
[4]Average coverage (%) of dominant species measured by the random sampling method with 20 microplots, transferred to plots (10x10 m)
[5]Average coverage (%) of dominant species measured by microcoenose sampling method transferred to plots (10x10 m)
*statistically significant difference (T-test, independent variables), $p<0.05$

The number of recorded species using the microcoenose sampling method was higher in comparison to the random sampling method, and there was no statistical significant difference ($p<0.05$) between the actual and recorded species number at each studied site (Table 3)

Coverage of dominant species

The dominant species were: *Abietinella abietina* (Hedw.) M. Fleisch. at V and IS, *Brachytheciastrum velutinum* (Hedw.) Ignatov & Huttunenat D and *Dicranum scoparium* Hedw. at PD. The coverage of dominant species obtained by the microcoenose sampling method showed no significant difference ($p<0.05$) when compared with the actual coverage of dominant species on the studied plots, while random sampling in both cases showed significantly different dominant species coverage in comparison to its actual coverage (Table 4).

Diversity index

The Shannon diversity index for 10x10 m plots showed different values for different sampling methods at each locality. The microcoenose sampling method showed the highest diversity index (Fig. 3A) at all tested sites. For V, D, IS and PD, the Shannon

diversity index values were 2.836, 3.236, 2.801 and 2.947, and for evenness 0.7408, 0.7265, 0.5876 and 0.9067, respectively (Fig.3B). The Shannon diversity indices for V, D and PD obtained using both random sampling methods showed statistically significant differences ($p<0.05$) when compared to the indices calculated from the results obtained by the microcoenose sampling method. One exception was the IS locality, where no significant difference in the Shannon index was found among the three sampling methods.

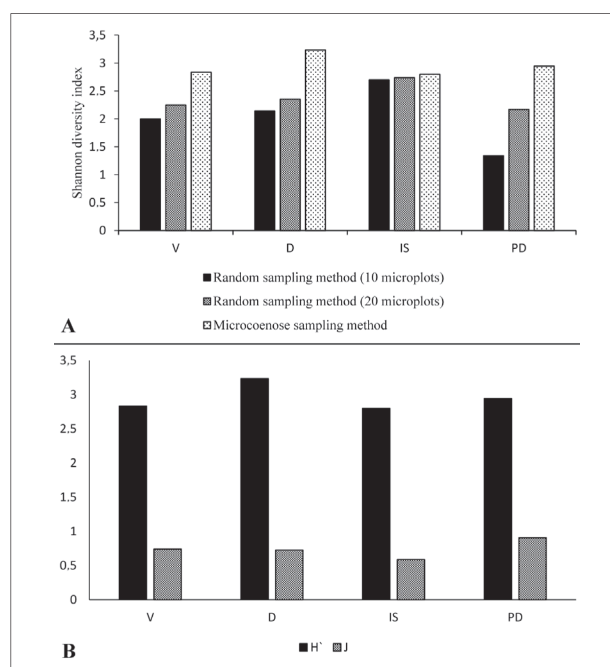

Fig. 3. Shannon diversity index and evenness. **A** – Shannon diversity index for each studied site (V – Vrdnik; D – Dumbovo; IS – Iriški Venac-Stražilovo; PD – Papratski Do) in relation with different sampling methods; **B** – Shannon diversity index (H`) and evenness (J) calculated from the microcoenose sampling method for each locality transferred to plot size 10x10 m.

Sampling time

The time needed for sampling was calculated only for locality D (Table 5). The average time per plot (10x10 m) was similar for random sampling with 20 microplots and microcoenose sampling, because they involved a similar number of microplots per plot. Statistical analysis showed that there was a significant difference ($p<0.05$) in average time between random sampling with 10 microplots and both of the other sampling methods, while the random sampling method with 20 microplots and the microcoenose method were not significantly different.

Table 5. Time* needed for sampling using different sampling methods at the Dumbovo site.

	Random sampling (10 microplots)	Random sampling (20 microplots)	Microcoenose sampling
Plot 1**	125	198	201
Plot 2**	110	245	275
Plot 3**	102	305	298
Plot 4**	114	275	304
Plot 5**	78	264	286
Average	105.8	257.4	272.8

*Time in min
**Plot size 10x10 m

DISCUSSION

Selection of efficient sampling methods for ground bryophytes in forests is very important for understanding bryophyte diversity and distribution patterns. Because of previously mentioned problems in quantifying bryophytes in forest ecosystems, an appropriate method should be maximally representative and applicable for a range of research tasks, while at the same time simple and time effective. Moreover, determination of the minimum sampling size remains very important, due to the high correlation between sample size and representativeness.

In the present work, ground bryophytes were studied quantitatively based on the minimum area concept. Quadrat size was determined using species-area curves. This method for estimation of minimal area is commonly used, and often recommended, but remains highly problematic [30]. The main problem with species-area curves is that species richness rarely reaches complete saturation [44], as was the case in the present study. Using standardized plot sizes increases the possibility of comparing different studies [28], but this is not always appropriate for forest bryophytes. Bryophytes are very sensitive to differences in the forest microenvironment [45], and they therefore often have a patchy distribution, as was found in the present study. In fact, the actual coverage of bryophytes was similar for three study sites (V, D and PD), while it was higher for locality IS. A possible reason for this pattern may be the poorly developed herbaceous layer

we observed at the IS site. Saetersdal et al. [46] demonstrated a high correlation between herbaceous flora and the development of a bryophyte layer. At the other three sites (V, D and PD), bryophytes had a strictly patchy distribution. The reasons for this patchy distribution are numerous and include light, temperature, water balance, poor competitiveness in comparison to vascular plants, and reproduction strategies [47].

Vanderpoorten et al. [7] strongly recommend the determination of sample quadrats by species-area curve, due to the fact that using small quadrats may lead to the exclusion of some important species, while unconventionally large quadrats can exclude important microhabitats. The minimum area concept in the present study showed that an appropriate minimum area for sampling ground bryophytes at all tested sites was 50x50 cm. Jiang et al. [32] determined the same minimum sampling size using the same methodology for broadleaved and mixed forests in China. This subplot size was also used for studies of boreal swamp forests [23] and broadleaved forests in China [26]. Different studies of forest-floor bryophytes showed variations in plot size, indicating the application of a plot size smaller than 0.25 m^2. Vellak and Paal [20] concluded that the appropriate plot size for boreal and boreo-nemoral forests in Estonia was 0.2x0.2 m due to the uniform microhabitat conditions. Økland [48] set the critical limit of representativeness for spruce forest plots in Norway at 0.01 m^2. Rambo and Muir [19] used 10x30 cm microplots for investigating *Pseudotsuga menziesii-Tsuga heterophylla* forests in Oregon, while Steel et al. [1] used quite small microplots of only 0.1x0.1 m. Small microplots are also used in studies of epiphytic bryophytes. For example, the sizes of microplots in corticolous bryophytes in several studies were 0.0225 m^2 [49,50], 0.1 m^2 [51], 0.0004 m^2 [52] and 0.04 m^2 [53]. Microplots larger than 0.25 m^2 are usually used in studies that deal with vascular plants [54] as well as vascular plants and ground bryophytes [21]. In general, the minimal sampling size is strongly dependent on the different types of ecosystems and the aims of the research.

The two sampling methods tested in the present study are different with respect to recorded species richness. Based on the species richness, the random sampling method using 10 microplots per plot (10x10 m) was found to be inadequate for researching ground bryophytes in temperate beech forests. At all tested localities, this method produced a significantly different (smaller) number of species than the actual species numbers in the plots. One possible reason for this is the structure of the ground bryophyte flora. At V, PD and D sites, the ground bryophytes had a patchy distribution, while, at the IS locality, the total coverage of bryophytes on the forest floor was greater than 90%. Consequently, the patchy distribution of ground bryophyte flora resulted in varying numbers of microplots without bryophytes, which is problematic during analyses of only bryophyte flora that exclude other plants (e.g. vascular herbaceous flora). The same pattern was observed after doubling the number of microplots (to 20), while an exception was the IS site, where bryophytes had a total coverage greater than 90% in 10x10 m plots. The main disadvantage of random sampling in these forests is the number of microplots without bryophytes. At each studied site where bryophytes had a patchy distribution there were several microplots without bryophytes. In order to achieve complete randomness – by randomly throwing a wooden frame (minimum area quadrat) – it is impossible to predict whether it will fall on an area with bryophytes, because of their patchy distribution.

Higher numbers of microplots per plot for random sampling led to increased values for measured species richness. Based on species richness, it was concluded that 10 or 20 quadrats 50x50 cm size were not sufficient for an investigation of ground bryophyte flora in the studied forest because the bryophytes had a patchy distribution. It is possible that increasing the number of microplots might lead to greater representativeness, although this would be quite time consuming. In general, random sampling is recommended to reduce the chance of bias, and the main advantage of complete plot randomization is that samples can be considered to be independent [55]. In the "ideal" case, random samples would not overlap, but in practice this can happen, which could be problematic for the interpretation of results [41]. The microcoenose sampling method showed much better results in terms of species richness for the present study sites, because there was no significant difference between the measured and actual species numbers. The results from the present study were in agreement with previous

investigations. Jiang et al. [32] reported similar results, following the application of this method for investigating ground bryophyte flora in broadleaved forests in China.

Coverage by dominant species measured at microplots and transferred to 10x10 m plots, showed significantly smaller dominant species coverage vs. actual values in comparison to the microcoenose sampling method.

Microcoenose sampling showed the highest Shannon diversity indices for all studied sites when compared to random sampling due to the fact that the highest species numbers were recorded using this method. Locality IS showed a similar diversity index compared to the other studied sites; however, its relatively low evenness index indicates the presence of one dominant species. In contrast, the PD site showed the highest evenness index due to an almost even participation of all species in ground bryophyte flora. For localities V, D and PD, the Shannon diversity indices obtained by the random sampling method (with 10 and 20 quadrats) were significantly different (for $p<0.05$) as compared to the Shannon diversity indices obtained by microcoenose sampling. At the IS study site, the situation was quite different, as there were no statistically significant differences between the Shannon diversity indices obtained by all three sampling methods. The most likely reason for this is the patchy distribution of ground bryophytes at the first three localities: as previously mentioned, the random sampling method appears to be inadequate for ground bryophyte flora in heterogeneous habitats. The second reason is the significant dominance of one species at the IS site.

Regarding sampling time, some form of systematic sampling is often recommended over random sampling [45,56]. In our study, there were no significant differences between the microcoenose and random sampling methods with 20 microplots. Increasing microplot numbers in order to achieve complete succession of random sampling will increase sampling time. Because of this, we consider the microcoenose sampling method to be more time effective in comparison to random methods.

CONCLUSIONS

The present study focuses on the comparison of microcoenose and random sampling methods, in order to define an appropriate method for sampling ground bryophyte flora in temperate forests. Based on the analysis of our findings, we concluded that the microcoenose sampling method is highly applicable in temperate beech forests as regards species richness and diversity. Furthermore, microcoenose sampling is quite time effective in comparison to random sampling. The application of this method for research that includes other plants (herbaceous flora) in forest stands should be tested. The validity of this method for adoption of statistical inferences regarding the whole community remains to be determined.

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Supplementary Data

Supplementary Figs. S1-S4 available at: http://serbiosoc.org.rs/sup/2869_Supplementary.pdf