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A nonparametric approach in quantifying species richness of Lumbricidae in East Serbia, Balkan Peninsula

Mirjana STOJANOVIĆ, Jovana SEKULIĆ, Tanja TRAKIĆ*
Institute of Biology and Ecology, Faculty of Science, University of Kragujevac, Kragujevac, Serbia

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Abstract: The concept of species richness is currently the most basic and most often used parameter in quantitative assessments of biodiversity. The species richness of Lumbricidae was investigated in East Serbia, one of the highly complex areas in the eastern and central part of the Balkan Peninsula. Our database included 2615 individuals from a total of 54 species. Quantification of species richness was done by using the observed number of species and richness estimators. A nonparametric approach was used to evaluate the performance of various estimation techniques: Chao 2, Jackknife 2, and Bootstrap. However, only Chao 2 reached the asymptote, maintaining values of 77 until the end of the curve. A total of 70.12% of the estimated number of Lumbricidae living in East Serbia were detected in our field study and we should expect that 23 lumbricid taxa will be added to the inventory in the future. The sampling effort needed to find additional species undetected during the sampling is 214 individuals. Chao 2 generally outperforms other estimators since it attains near-asymptotic stability and, therefore, more accurate prediction of earthworm species richness.

Key words: Lumbricidae, species richness, estimators, Balkan Peninsula

1. Introduction

The concept of species richness is currently the most basic and most often used parameter of many field studies carried out in community ecology (Gaston, 1996, 2000; Whittaker et al., 2001). However, there are many issues regarding its measurement. Importantly, the richness of species in a community usually cannot be observed directly because a complete enumeration of every species is infeasible. In most empirical studies, the observed number of species is used as a surrogate for the true number of species. However, the observed number of species typically excludes many rare species and seriously underestimates the true number of species (Colwell and Coddington, 1994). Many extrapolation methods have been developed to reduce this bias (Colwell and Coddington, 1994; Colwell, 2006).

Bearing in mind that species richness is a central component of biodiversity, the maximization of species richness represents one of the most important goals for their maintenance, which is clearly noticeable in the case of Lumbricidae in the area of the Balkan Peninsula. It is known that the Balkan Peninsula is one of the most important biodiversity hotspots in Europe (Griffiths et al., 2004). This is the result of frequent changes in global ecological conditions, which have greatly contributed to the occurrence of an exceptionally heterogeneous fauna (Mršić, 1991; Džukić and Kalezić, 2004).

The first studies on earthworms from the Balkan Peninsula were published by Rosa (1897) and Cognetti (1906). Mršić (1991) listed about 200 species and subspecies registered for the territory of the Balkans and neighboring countries (57 from Serbia, 68 from Slovenia, 59 from Croatia, 47 from Macedonia, 45 from Bosnia-Herzegovina, 36 from Montenegro). Following this comprehensive work, the more recent data are mainly from Serbia (Stojanović and Karaman, 2003, 2006, 2007; Stojanović et al., 2008, 2013; Milutinović et al., 2010; Szederjesi and Csuzdi, 2012a), Montenegro (Stojanović and Karaman, 2003; Stojanović and Milutinović, 2013; Szederjesi, 2014), Croatia (Hackenberger and Hackenberger, 2013, 2014), and Bulgaria (Stojanović et al., 2012, 2013; Valchovski, 2014). There have been very few works from Greece (Szederjesi and Csuzdi, 2012b), Macedonia (Mršić, 1991), Albania (Szederjesi and Csuzdi, 2012a), and Bosnia-Herzegovina (Szederjesi, 2013).

The aims of this paper are to establish the current fauna composition of Lumbricidae and determine the species richness within the area of East Serbia (Balkan Peninsula), to evaluate alpha diversity with species observed curves as a function of the accumulated number of individuals and species richness estimates, and to assess the performance of various estimation techniques to determine the

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* Correspondence: tmilutinovic@kg.ac.rs

efficiency of the different species richness estimators, using a nonparametric approach (Chao 2, Jackknife 2, and Bootstrap). Taking into account that there are limited data in the literature on the implementation of these methods on earthworm species richness estimation, we have tried to follow a methodological and theoretical framework for the application of species richness estimators in soil invertebrate biodiversity research.

2. Materials and methods

2.1. Study area

It is known that the natural world of the Balkan Peninsula has gone through a very dynamic history, throughout which it has changed and gained a remarkable complexity of abiogenic and biogenic factors that, with their variation over a tumultuous geological history, define the current environmental framework of its biodiversity. Therefore, landscape heterogeneity is a habitual feature in the Balkan Peninsula, especially in East Serbia, which has always attracted attention because of its vast diversity of landforms and species (Lopatin and Matvejev, 1995; Savić, 2008).

Located in the border territory of the eastern and central Balkan Peninsula, East Serbia (42°53′–44°43′N, 21°30′–23°00′E) covers the area between the Danube in the north and the Stara Planina Mountains (Balkan Mountains) in the southeast, along the Timok Valley. The position of East Serbia as well as its exposure to varying climatic influences, modified by complex mountain projections and various petrographic and edaphic conditions, are the major abiotic factors contributing to the great biodiversity of its territory (Savić, 2008).

The eastern part of Serbia is a hilly-mountainous area with a moderate continental steppe climate (Savić, 2008; Jakšić, 2008). The basic geological mass of the Balkan mountain system in East Serbia is the western part of the Stara Planina Mountains, joined by many of the mountains of East Serbia (Vlach Mountain, Ozren Mountain, and Rtanj Mountain). The western Stara Planina Mountains are an extension of the Carpathian mountain range and stretch in a north–south direction in East Serbia, east of the Morava Valley. In orographic and geomorphological terms, this area is extremely heterogeneous and complex. The Carpatho-Balkan mountain system is composed of ancient metamorphic limestone with a predominance of vertisols and metamorphic red rendzina, terra rosa, and brown soils. On the proposal of the Institute of Nature Protection of Serbia, in 1997 the Western Stara Planina Mountains were placed under strict protection as having "natural merit of first class". They are also the object of an agreement of cooperation between the ministries of Bulgaria and Serbia for the formation of a transboundary protected area. In addition, due to their geographic position and paleogeographic history, the western area of the Stara Planina Mountains should be considered as a diversity hotspot in the Balkans and should merit designation as a Transborder Important Area (Papp and Erzberger, 2007; Jakšić et al., 2011).

Along the right bank of the Danube River, through the southern slopes of the Carpathian Mountains, there is another important area of East Serbia: Đerdap National Park, formed by 4 successive gorges separated from each other by ravines. Moreover, 11 nature reserves are present in East Serbia as well. East Serbia represents an interesting mosaic on the European biogeography map. Horvat et al. (1974) classified 7 vegetation zones in the Balkan Peninsula. Each zone contains a great number of different biotopes (Lopatin and Matvejev, 1995). In our study, 2 habitat types were defined: grassland and woodland (deciduous forests: oak, beech, and different mixtures).

2.2. Lumbricidae sampling

This study of earthworms was obtained from fieldwork based on 840 samples from 28 localities (30 samples from each of the habitats, over the course of 1 year). The specimens were obtained by the diluted formaldehyde method complemented with digging and hand-sorting, turning over rocks, debris, and logs. For each sample (40 × 40 cm), 10 L of 4% formalin was used. In 90% of the samples per habitat, the formalin method was used; 10% of the samples were obtained by searching rocks, debris, and logs. The earthworms were killed in 70% ethanol, fixed in 4% formalin solution, and stored in 90% ethanol. Identification of species was made in accordance with Csuzdi and Zicsi (2003), Blakemore (2008), and Mršić (1991).

2.3. Data analysis

The observed number of species and nonparametric estimators were included in the study to estimate species richness. We evaluated alpha diversity with species observed curves as a function of the accumulated number of individuals or samples (Colwell and Coddington, 1994) and species richness estimates (Figure). Observed species richness is the most obvious measure of alpha diversity, but it is clear that, due to the presence of rare species, the observed species accumulation curves underestimate species richness. Thus, a number of estimates have been developed to predict true species richness (Chazdon et al., 1998). Sample-based data were used for the calculation of the richness estimators, using EstimateS Version 8 (Colwell, 2006). Chao 2, Jackknife 2, and Bootstrap were used to determine the efficiency of nonparametric richness estimators. The Chao 2 (Chao, 1987) and second-order Jackknife (Smith and van Belle, 1984; Palmer, 1991) estimators clearly provide the least biased estimates for small numbers of samples (Colwell and Coddington, 1994). On the number of individuals per sample, Chao 2 does not require precise information. According to

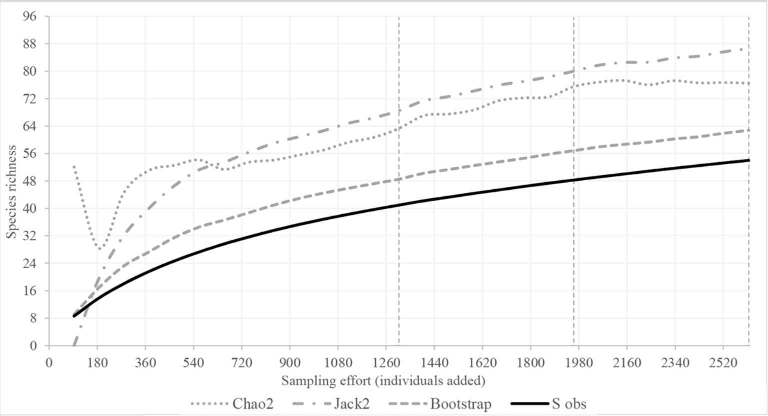

Figure. Observed (S obs) and estimated species richness for Chao 2, Jackknife 2, and Bootstrap, calculated for Lumbricidae in East Serbia. Vertical dashed lines represent 50%, 75%, and 100% of the sampling effort, respectively.

Chazdon et al. (1998), as an incidence-based estimator of species richness, Chao 2 relies on the number of species found in only 1 unit (unique units) and 2 sample units (duplicates).

The second-order Jackknife estimator, similar to Chao 2, is based on the numbers of unique units and duplicates and the number of sampled units. The Bootstrap (Smith and van Belle, 1984) estimator is based on the proportion of quadrats containing each species, and thus it requires only incidence (presence/absence) data.

In order to determine the slope of the end of the curves, we statistically checked whether the randomized curves were increasing, decreasing, or approaching the asymptote by the end of the sampling process. The slope value is the inverse of the number of individuals that must be collected for the purpose of increasing the species count by 1. For the end of the curve, slope values close to 0.001 can be considered as having reached the asymptote. To change the value of species richness, more than 1000 individuals would be expected to be needed.

Based on the formula from Cardoso et al. (2008), the final slopes of curves were calculated:

Slope = 1 / (ns – ns ± 1),

where ns = final number of individuals for each curve (total richness value S) and ns ± 1 = number of individuals corresponding to the point on the curve where the final single species was added or subtracted to S (richness value of S ± 1).

Sampling intensity, defined as the ratio of specimens to species, was calculated as a measure of sampling effort (Coddington et al., 1996). Sufficient sampling effort for reaching the asymptotic species richness obtained by Chao 2 can be calculated by using the formula provided by Chao et al. (2009), which is based on the presence of species that occur in exactly 1 sampling site, as well as the species that occur in 2 sampling sites (unique units and duplicates sensu) (Colwell and Coddington, 1994).

For analyzing the abundance patterns in the study area, we applied the dominance analysis proposed by Tischler (1949), with small changes (Pešić, 1997). The dominance index was calculated according to the following formula:

$$Di=(a1/\sum_{i=1}^{n} a2)\times 100$$

where Di is the dominance of a certain species, $a1$ the number of specimens of that species, and $a2$ the total number of adults of all species. According to the calculated values, species were classified into 5 common categories: eudominant (ED: >10%), dominant (D: 5.1%–10%), subdominant (SD: 2.1%–5%), recedent (R: 1.1%–2%), and subrecedent (SR: ≤1%). In the text that follows, we used the abbreviations ED, D, SD, R, and SR, respectively.

3. Results

3.1. Estimate of species richness

Fifty-four earthworm species were collected from the eastern part of Serbia (Table 1). The Figure shows the curves of observed species richness and several estimators. The observed richness for Lumbricidae (S = 54) is not far from those of the nonparametric estimators Bootstrap (63), Chao 2 (76), and Jackknife 2 (87). The richness estimates indicated that more than 62% of the taxa were collected in East Serbia (Table 2). The Chao 2 estimator showed a slow climb to its steady value of 77 species from 21 localities. However, only Chao 2 reached the asymptote (Figure), maintaining the value of 77 until the end of the curve. The slope value is close to 0.001. Therefore, the sampling effort needed to find an additional 23 species undetected during the sampling is 214 individuals. On the

Table 1. List of Lumbricidae recorded in East Serbia indicating their total abundance and their level of dominancy (ED- eudominant, D- dominant, SD- subdominant, R- recedent, and SR- subrecedent).

Taxa	Total	Dominancy
Genus *Allolobophora* Eisen, 1874		
Allolobophora chlorotica (Savigny, 1826)	10	SR
Allolobophora dofleini (Ude, 1922)	26	SR
Allolobophora kosowensis kosowensis Karaman, 1968	5	SR
Allolobophora leoni Michaelsen, 1891	52	R
Allolobophora mehadiensis Rosa, 1895	1	SR
Allolobophora paratuleskovi Šapkarev, 1975	3	SR
Allolobophora robusta spasenijakaramani Blekmore, 2004	11	SR
Allolobophora serbica (Šapkarev, 1977)	2	SR
Genus *Aporrectodea* Örley, 1885		
Aporrectodea caliginosa (Savigny, 1828)	21	SR
Aporrectodea georgii (Michaelsen, 1890)	4	SR
Aporrectodea handlirschi (Rosa, 1897)	53	R
Aporrectodea jassyensis (Michaelsen, 1891)	24	SR
Aporrectodea macvensis (Šapkarev, 1987)	3	SR
Aporrectodea rosea leocernosvitovi (Blakemore, 2004)	49	R
Aporrectodea rosea rosea (Savigny, 1826)	735	ED
Aporrectodea sineporis (Omodeo, 1952)	28	R
Aporrectodea smaragdina (Rosa, 1892)	9	SR
Aporrectodea sturanyi (Rosa, 1895)	4	SR
Aporrectodea trapezoides (Duges, 1828)	99	SD
Genus *Cernosvitovia* Omodeo, 1956		
Cernosvitovia getica (Pop, 1947)	1	SR
Cernosvitovia biserialis (Černosvitov, 1938)	1	SR
Cernosvitovia opisthocystis (Rosa, 1895)	9	SR
Genus *Dendrobaena* Eisen, 1874		
Dendrobaena attemsi (Michaelsen, 1902)	26	SR
Dendrobaena byblica (Rosa, 1893)	130	SD
Dendrobaena illyrica (Cognetti, 1906)	3	SR
Dendrobaena hortensis (Michaelsen, 1890)	42	R
Dendrobaena jastrebensis (Mršić & Šapkarev, 1987)	61	SD
Dendrobaena kozuvensis (Šapkarev, 1971)	3	SR
Dendrobaena octaedra (Savigny, 1826)	60	SD
Dendrobaena veneta (Rosa, 1886)	15	SR
Genus *Dendrodrilus* Omodeo, 1956		
Dendrodrilus rubidus rubidus (Savigny, 1826)	2	SR
Dendrodrilus rubidus subrubicundus (Eisen, 1874)	6	SR
Dendrodrilus rubidus tenuis (Eisen, 1874)	13	SR
Genus *Eisenia* Malm, 1877		
Eisenia foetida (Savigny, 1826)	107	SD
Eisenia lucens (Waga, 1857)	136	D

Table 1. (Continued).

Genus *Eiseniella* Michaelsen, 1900		
Eiseniella tetraedra pupa (Eisen, 1874)	33	R
Eiseniella tetraedra tetraedra (Savigny, 1826)	27	SR
Genus *Fitzingeria* Zicsi, 1978		
Fitzingeria platyura platyura (Fitzinger, 1833)	R	33
Genus *Helodrilus* Hoffmeister, 1845		
Helodrilus balcanicus balcanicus (Černosvitov, 1931)	1	SR
Helodrilus balcanicus plavensis (Karaman, 1972)	2	SR
Helodrilus cernosvitovianus (Černosvitov, 1931)	51	R
Genus *Lumbricus* Linnaeus, 1758		
Lumbricus improvisus (Zicsi, 1963)	4	SR
Lumbricus castaneus (Savigny, 1826)	6	SR
Lumbricus polyphemus (Fitzinger, 1833)	19	SR
Lumbricus rubellus Hoffmeister, 1843	390	ED
Lumbricus terrestris (Linnaeus, 1758)	2	SR
Genus *Octolasion* Örley, 1885		
Octolasion cyaneum (Savigny, 1826)	2	SR
Octolasion lacteum lacteum (Oerley, 1881)	279	ED
Genus *Octodrilus* Omodeo, 1956		
Octodrilus bretscheri (Zicsi, 1969)	1	SR
Octodrilus complatanus (Duges, 1828)	1	SR
Octodrilus transpadanus (Rosa, 1884)	6	SR
Genus *Perelia* Easton, 1983		
Perelia nematogena (Rosa, 1903)	1	SR
Genus *Proctodrilus* Zicsi, 1985		
Proctodrilus antipai (Michaelsen, 1891)	4	SR
Proctodrilus tuberculatus (Černosvitov, 1935)	74	SD

other hand, Jackknife 2 (slope 0.002) and Bootstrap (slope 0.003) were still rising at the end of the curves. Unlike the other estimators, Chao 2 stabilized upon estimating 77 species. Consequently, the great advantage of using Chao 2 is confirmed by our research.

3.2. Abundance patterns

Altogether, a total of 2615 specimens of Lumbricidae were collected in East Serbia. *Aporrectodea rosea* was the most abundant species. This species represented 30% of all the individuals collected. *Lumbricus rubellus* was the second most abundant species. According to the Tichler (1949) dominance scale, there are 3 eudominant species (*A. rosea*, *Lumbricus rubellus*, and *Octolasion lacteum*) in the study area (Table 1). Furthermore, in East Serbia, there are 1 dominant species, 6 subdominant species, 12 rare species, and 8 recedent species (Table 1). The greatest number of species (36) belong to the subrecedent taxa (<1%).

4. Discussion

East Serbia is a highly complex area in the eastern and central part of the Balkan Peninsula. Several circumstances have contributed to its complexity. First, in the eastern area of Serbia, there is the Carpatho-Balkan mountain system composed of complex geological substrates in combination with microorographic, hydrological, and climatic conditions.

Another interesting peculiarity contributes to the unique richness in East Serbia, according to Jakšić et al. (2011), who emphasized the importance of the distribution of carbonate rocks to the biodiversity of the Balkan Peninsula. Bearing in mind a clear demarcation in the distribution of carbonate rocks of the carbonate platform terrane and tectonic units, as well as the fact that these units have a genuine relict flora and fauna, Jakšić et al. (2011) developed a biogeographical theory (the concept

Table 2. Observed (S_{obs}) and estimated species richness and percentage of the estimated value of Lumbricidae in East Serbia based on nonparametric methods.

Species richness estimator*	
Species observed	54
Individuals	2615
Chao 2	76.5
Jackknife 2	86.6
Bootstrap	62.77
Singletons	7
Doubletons	5
Unique units	21
Duplicates	8
Completeness	64%–88%

* Completeness is a percentage of estimated richness of Lumbricidae (minimum–maximum).

of anchored terrane) that could explain the origin, genesis, and great biodiversity in the eastern part of Serbia. On the other hand, the complex geological history of this area together with drastic climatic changes in the past have contributed to the development of highly complex and heterogeneous soil fauna. These changes have made East Serbia a center of diversification for many groups of organisms (Džukić and Kalezić, 2004; Makarov et al., 2004). In addition, the eastern part of Serbia (together with the mountainous parts of Bulgaria) has been declared a European center of biodiversity (Jakšić, 2008).

4.1. Nonparametric estimator performance

Species richness is an important characteristic of ecological communities, but it is difficult to quantify. For this purpose, the use of nonparametric estimators has been suggested by many authors. Based on evaluating the performance of 8 nonparametric richness estimators on a seed-bank data set, Colwell and Coddington (1994) suggested the use of Chao 2 and Jackknife 2 due to the fact that these estimators provided the least biased estimates for small numbers of samples. In a study of seedling and sampling diversity, Chazdon et al. (1998) found that ICE and Chao 2 were robust for sample size and patchiness. In a study of carabid beetles in the East German agricultural landscape, Brose (2002) estimated species richness by observing the number of species and using nonparametric estimators (Chao 2, Bootstrap, Jackknife 1, and Jackknife 2). He concluded that Chao 2 appeared to be more precise compared to the other estimators used.

Recently, Dey and Chaudhuri (2013) used 6 different nonparametric richness estimators (Chao 1, Chao 2, ACE, ICE, Jackknife 1, and Jackknife 2) in comparative analysis of earthworm species richness between 2 types of plantations in India. They recommend that the Chao 2 richness estimator be applied. Similar results were obtained in a study on earthworms from Croatia by Hackenberger and Hackenberger (2014). They evaluated the efficiency of sampling based on 7 nonparametric species richness estimators. They concluded that the most inadequate nonparametric estimators appeared to be Jackknife 1 and Jackknife 2, while Chao 1 and Chao 2 were the most suitable of all calculated estimators. Milutinović et al. (2015) evaluated the performance of 8 different nonparametric richness estimators (ACE, ICE, Chao 1, Chao 2, Jackknife 1, Jackknife 2, Bootstrap, and Michaelis–Menten). They concluded that the Chao 2 richness estimator is the most appropriate to predict the number of earthworms and can be used to control for the confounding effects of sampling effort in studies of earthworm species richness.

Based on our results, when comparing the performance of 3 different species richness estimators, it can be observed that Chao 2 generally outperforms other estimators, since in 1 of 3 distinct faunal samples it attains near-asymptotic stability. The sampling effort for the attainment of a near-stable asymptote is, however, around 80% of the total effort, suggesting that the collected sample size can only be slightly lower than the size proposed in this study for Lumbricidae. Since the value of Chao 2 is dependent on the distinctiveness of sampling sites (the number of unique units and duplicates) (Unterseher et al., 2008), it can be argued that the diversity of Lumbricidae has an influential spatial component characterized by the presence of locally abundant, but otherwise rare, species. Therefore, an increase in the diversity of sampling sites can probably lower the overall sampling effort for the accurate richness estimate and also increase completeness of the species inventory achieved by Chao 2, since the species that were not discovered in the sample are estimated from the proportional abundance of species within the total sample (Colwell and Coddington, 1994). The pronounced presence of rare species within the sample predicts encountering more new species with increased sampling effort (Gotelli and Colwell, 2010). Chao 2 indicates a specific pattern of species richness represented by the presence of several widespread species along with locally rare ones. However, to know the total richness in rich communities with many rare species, an unfeasibly large sample may be needed. Moreover, even the best performing estimator requires a certain sampling effort before stable values are calculated for a given habitat or site of interest.

In conclusion, the species-richness estimates from our current data set indicate that we should expect that 23 lumbricid taxa will be added to the inventory in the future in the eastern part of Serbia. The most adequate nonparametric estimator in the richness research of Lumbricidae appeared to be Chao 2, in the sense of reaching the asymptote first of all calculated estimators; therefore, Chao 2 can provide a quantitative basis for assessing long-term changes in species richness earthworm studies.

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