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ESTIMATING SUBTERRANEAN SPECIES RICHNESS USING INTENSIVE SAMPLING AND RAREFACTION CURVES IN A HIGH DENSITY CAVE REGION IN WEST VIRGINIA

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Species richness in a group of caves in the 21.25 km² corner of the USGS 7 ½ minute Williamsburg quadrangle, West Virginia, was investigated to (1) increase our knowledge of species richness for this area, (2) determine how many caves need to be sampled to achieve an accurate estimate of species richness and (3) estimate how many species are present in this area. Eighteen subterranean invertebrate species were collected from 65 caves within the study area. Seven caves were needed to collect 95% of the species. By sampling only the largest seven caves, 89% of the species were captured. However, the species accumulation curve did not reach an asymptote, and estimations based on species rarity show that half of the species were not collected at all. Therefore, the observed patterns should be interpreted with caution, and more data are needed.

Biological sampling and biodiversity mapping have become key components to the understanding of subterranean ecosystems in the face of environmental and anthropogenic threats (Culver *et al*. 2001), and promoting the assessment of the status and vulnerability of cave species facilitates their preservation and protection. Mapping biodiversity is an important step in this endeavor, serving as a tool for education, research, and conservation planning (Culver *et al*. 2001).

The information incorporated into maps of species richness in caves can come from a variety of sources, such as inventory or census information or from known occurrence records (Conroy & Noon 1996). The accuracy of these maps and eventual protection of biological diversity therefore hinges on the completeness of these data (Kodric-Brown & Brown 1993, Keating *et al*. 1998). However, there is an inherent bias in relying on occurrence records and compiled lists, in that most of these lists are incomplete and not all caves have been carefully and repeatedly studied, if they have been studied at all. In addition, sites that have been sampled but in which no species were found are typically not displayed on biodiversity maps, making them indistinguishable from unsampled sites (Deharveng 2001).

Sampling incompleteness can result in misleading patterns in community structure and species rarity, as Kodric-Brown and Brown's (1993) study of the effect of different levels of sampling of fish species richness in Australian desert springs shows. This is often compounded by sampling bias towards accessible sites, such as cities and highways (Bojórquez-Tapia *et al*. 1994) and field stations (Pearson & Cassola 1992), as well as bias towards certain taxa (Bojórquez-Tapia *et al*. 1994), that affect the reliability of occurrence data (Bojórquez-Tapia *et al*. 1995). As a consequence of incomplete sampling, not all species may be represented, leading to inaccurate estimates of species richness (Nichols *et al*. 1998), and possible poor decision making in conservation planning and management (Conroy & Noon 1996). Inventories of subterranean fauna may be so inadequate that many species may go extinct before being discovered (e.g., Croatia [USAID 2000]).

Thus far, richness estimates for cave faunas have been derived based on extrapolation from a small number of well-studied caves, which often tend to be the largest and most accessible (Culver *et al*. in press). It is unclear how inaccurate and/or misleading our knowledge of subterranean biodiversity may be. To date, no cave area has been sampled completely, except possibly for the Canary Islands (Izquierdo *et al*. 2001). The Derbyshire region of Britain has had 27% of the 210 caves sampled (Proudlove 2001) and may be the second most completely sampled region. In West Virginia, an area thought to be well-sampled (Culver & Holsinger 1992), less than 10% of the caves have been biologically sampled (Krow & Culver 2001), even though between 1962 and 1973, 152 caves were biologically investigated (Holsinger *et al*. 1976).

When variation between sites in species richness is great (as is the case for West Virginia caves), a larger sampling effort is required (Hammond 1994) to estimate total species richness. Sampling effort must be sufficient to minimize sampling bias in order to determine if inventory data are accurate (Hammond 1994). It is therefore critical that the sample of caves be large and unbiased (Krow & Culver 2001).

We sampled 65 caves within a high cave density and species-rich area of West Virginia. By sampling a large percentage of caves in an area, it is possible to discover how many species are missed when only a portion of the caves are sampled. We then used these data to examine how many caves need to be sampled to get an accurate estimate of species richness for the study area. Lastly, we predict how many species are indeed present in the study site using rarefaction curves and equations based on species rarity.

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STUDY SITE

West Virginia is reported to have 3754 caves (Jones 1997). Of these 195 (5.2%) are reported to have obligate cave-dwelling species (Culver, unpublished data). There are 76 known obligate cave species reported from the state (Culver & Sket 2000).

As far back as the 1950s, it was acknowledged that Greenbrier County, West Virginia, was rich in cave numbers, possessing some 105 caves — one quarter of all of the caves of West Virginia (Davies 1958). Today, the number of known caves from both locations has increased tenfold, with 1030 known caves from the county (Jones 1997). Greenbrier County is also a national hotspot of cave biodiversity (Culver *et al.* 2000).

An area centered around the Buckeye Creek Basin in northeastern Greenbrier County (Fig. 1) was chosen as the study site in part due to its high concentration of caves. The study site was chosen because of its high cave density on a limited number of properties, its proximity to the West Virginia Association of Cave Studies field station, and our good working relationship with the local landowners. We had access to all areas within the study area.

The invertebrate fauna of the study area was poorly known, and no systematic survey had been performed prior to this study (Fong & Culver 1994). Only 9 of the 148 caves previously had been biologically sampled, and 10 cave-limited species were reported from this study site (Holsinger *et al.* 1976, Fong & Culver 1994).

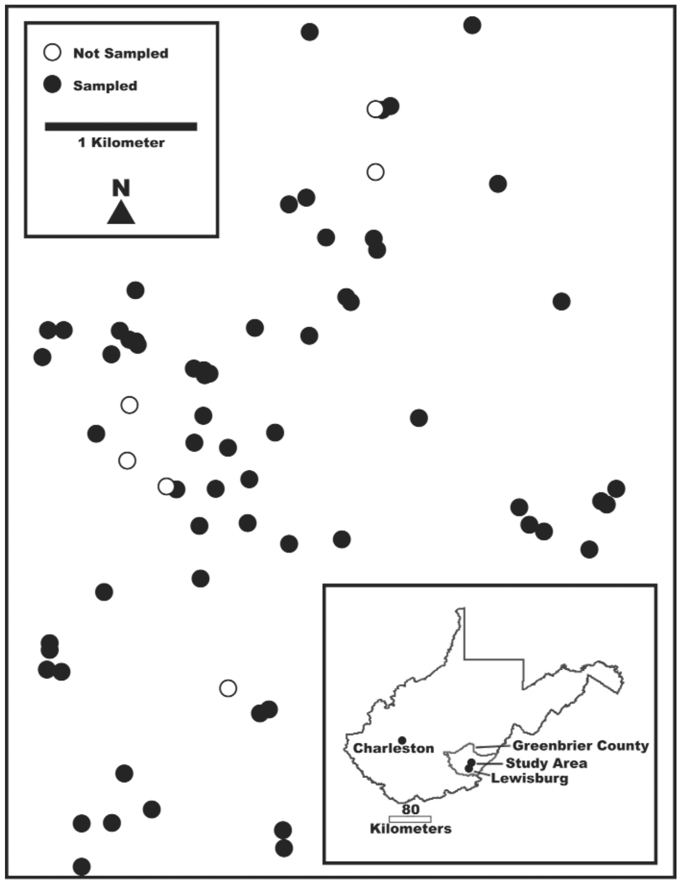

Figure 1. Locator map of the study site. The study site is a ca. 20 km² area located north of Lewisburg, in Greenbrier County, West Virginia. Of the 148 caves located in the study site, 73 were enterable. Of these 73 caves, 65 were sampled during this investigation.

METHODS

Cave locations were obtained from files of the West Virginia Speleological Survey (WVSS). The following criteria were used to select caves to be sampled. First, the caves must be located within the study area where 3' of latitude and 2'30" of longitude in the northeast corner of the USGS 7½ minute Williamsburg quadrangle was determined to be within the study area and representing an area of 21.25 km². Second, cave enterability was usually assessed by inspection and in some cases from the descriptions in Dasher and Balfour (1994).

Caves were located in the field using UTM coordinates, with a map provided by WVSS, and with the help of cavers and local residents. If more than two hours were spent unsuccessfully searching, the cave was classified as "failed to locate." Located caves were included in the study if they could be safely entered and had a dark zone. Entrances of study caves were then flagged and UTM coordinates recorded to facilitate relocating caves during the sampling period.

Sampling took place between June 3, 2002 and July 21, 2002. Once inside a cave, a visual census of organisms on the walls, floor, ceiling, and aquatic areas (if present) was performed for one-person hour, recording all species found. Only potential troglobites and stygobites were collected and these were placed in 70% ethanol. As a general rule, one to five specimens, an adequate number for positive identification to species, were hand-collected from each cave.

Terrestrial pitfall traps were constructed of 150 mL plastic jars filled with isopropyl alcohol and covered with 7.5 cm × 7.5 cm pieces of 1 cm hardware cloth to exclude cave-crickets, salamanders, and other larger animals. Pitfall traps were baited with limburger cheese and placed in soft mud banks, where mud banks were present. Aquatic traps were constructed of an ordinary kitchen scrubber with a mesh size of approximately 1 cm. The tube of mesh was baited with raw shrimp and tied at both ends. Aquatic traps were placed in slow-running shallow streams or rimstone pools. Traps were placed near areas of high abundance and diversity, as determined by visual sampling.

Generally, one terrestrial and one aquatic (if water was present) trap was placed in each cave. If the cave contained various habitats (e.g., rimstone pools and streams, mudbanks and silty shores, etc.), one to three additional traps of each type were placed. This was the case for most caves more than 100 m long. Traps remained in place for two to three days. Prior to trap removal, the surrounding area was examined and additional individuals attracted to the bait were collected. Animals from pitfall traps were transferred from isopropyl alcohol to ethanol in the laboratory.

Specimens were sorted and identified either by using keys or sending specimens to expert taxonomists. All species remained in ethanol except for the beetles which were transferred to Barber's fluid, a relaxant used to prevent brittleness and breakage of the specimens (Borror *et al*. 1989).

Maps were created using ArcMap GIS (Environmental Systems Research Institute, Redlands, CA, USA) and the UTM data were transformed using Datumpro (Linden Software Ltd, Lincs, United Kingdom). Data were analyzed using Excel (Microsoft Corporation, Redlands, CA, USA), SPSS (SPSS Inc., Chicago, IL, USA), and JMP (SAS Institute, Cary, NC, USA).

Rarefaction curves were made by repeatedly sampling all of the collected species at random (Gotelli & Colwell 2001). Rarefaction curves indicate the expected number of species from a collection of random samples and represent what is statistically expected from the accumulation curve (Gotelli & Colwell 2001). With rarefaction curves, differences are no longer attributed to sample size. Rarefaction curves were created using EstimateS software (Colwell, 1997; http://viceroy.eeb.uconn.edu/estimates).

Due to incomplete sampling, estimators have been derived to predict the true number of species based on rare species in a sample (Colwell & Coddington 1994). This was done using the equation from Chao (1984),

$$S_2^* = S_{obs} + \left(L^2 / 2M\right) \tag{1}$$

where *Sobs* is equal to the number of species observed in a sample, *L* is the number of observed species represented by a single individual (i.e., singletons), and *M* is the number of observed species represented by two individuals in the sample (i.e., doubletons).

The variance on this equation was estimated as

$$\operatorname{var}\left(S_2^*\right) = M\left[\left(\frac{L/M}{4}\right)^4 + \left(L/M\right)^3 + \left(\frac{(L/M)}{2}\right)^2\right] \tag{2}$$

Colwell and Coddington (1994) recommend the application of Burnham and Overton's (1978) jackknife estimators in order to reduce estimation bias in estimating species richness. We calculated this second-order jackknife estimate:

$$S_4^* = S_{obs} + \left[\frac{L(2n-3)}{n} - \frac{M(n-2)^2}{n(n-1)}\right] \tag{3}$$

where *n* is the number of samples. No direct formula for the calculation of the variance is available.

We used the algorithm of Csuti *et al*. (1997) to find the minimum number of caves needed to "capture" 95% of the reported troglobites and stygobites.

RESULTS

The WVSS (West Virginia Speleological Survey) database showed 148 caves, pits and FROs (for the record only) in the study site. We were able to locate and enter 65 of these caves in the summer of 2002 (Fig. 1). Of the remaining 83 locations, we were unable to locate a physically enterable entrance for 75 of them either because of faulty location data or because the WVSS database contained non-cave karst features. The eight additional enterable caves were located too late to be included in the study (January 2003), but are worth revisiting and sampling in future studies.

The average cave length was 165.3 m ±64.6 m and varied between 2 m and 3719 m. Most of the caves were short, with 44 of the 65 caves being less than 30 m long. Cave depth averaged 9.8 m ±1.4 m with a range of one to 30 m. Twenty-one of 33 caves for which depth data were available were less than 10 m deep. All caves had terrestrial habitats, but only 38 had aquatic habitats. An aquatic habitat was defined as an aquatic area in which a trap could be placed.

Overall, six classes, 11 orders, 12 families, 14 genera, and 18 species were collected (Table 1). The two most commonly encountered orders were the Collembola (springtails) and Coleoptera (beetles), followed by the Amphipoda (amphipods), Chordeumatida (millipedes), and Diplura (diplurans).

Three rarefaction curves are shown in Figure 2 — one for all caves, one for caves less than 15 m in length, and one for caves greater than 15 m in length. All three curves showed no sign of reaching an asymptote, but the rate of species accumulation for caves greater than 15 m was more than twice that of caves less than 15 m.

Two estimates of total species richness are provided in Table 2. The two estimates are 36 and 48, and both are considerably higher (between two and three times) than the observed number of 18.

Table 3 shows that seven caves are needed to find 17 of the 18 reported species and suggests which caves need to be sampled in order to collect 95% of the total species collected in the study. If the seven largest caves are used, the result is nearly as good, with 16 of the 18 reported species found in these caves, which implies that 89% of the total species are collected if the largest seven caves are sampled (Table 4). The largest caves themselves are not arranged in order of size but rather in order of their successive contribution of new species, so that in fact that last two caves added to the analysis do not result in the inclusion of any new species.

Table 1. Cave-limited species encountered during the study and their habitats.

Class	Order	Family	Species	Reference	Habitat
Turbellaria	Tricladida	Kenkiidae	*Macrocotyla hoffmasteri*[a]	(Hyman, 1954)	Aquatic
Mollusca	Gastropoda	Hydrobiidae	*Fontigens tartarea*[a]	Hubricht, 1963	Aquatic
Crustacea	Amphipoda	Crangonyctidae	*Stygobromus emarginatus*	(Hubricht, 1943)	Aquatic
Crustacea	Amphipoda	Crangonyctidae	*Stygobromus spinatus*	(Holsinger, 1967)	Aquatic
Crustacea	Isopoda	Asellidae	*Caecidotea holsingeri*	(Steeves, 1963)	Aquatic
Crustacea	Decapoda	Cambaridae	*Cambarus nerterius*	Hobbs, 1964	Aquatic
Diplopoda	Chordeumida	Cleidogonidae	*Pseudotremia* sp. nov.[a]	…	Terrestrial
Diplopoda	Chordeumida	Cleidogonidae	*Pseudotremia* sp.	…	Terrestrial
Insecta	Diplura	Campodeidae	*Eumesocampa* sp.[a]	…	Terrestrial
Insecta	Diplura	Campodeidae	*Litocampa fieldingae*[a]	(Condé, 1949)	Terrestrial
Insecta	Collembola	Sminthuridae	*Arrhopalites clarus*[a]	Christiansen, 1966	Terrestrial
Insecta	Collembola	Entomobryidae	*Pseudosinella gisini*	Christiansen, 1960	Terrestrial
Insecta	Collembola	Entomobryidae	*Sinella hoffmani*[a]	Wray, 1952	Terrestrial
Insecta	Coleoptera	Carabidae	*Pseudanopthalmus grandis*	Valentine, 1931	Terrestrial
Insecta	Coleoptera	Carabidae	*P. higginbothami*	Valentine, 1932	Terrestrial
Insecta	Coleoptera	Carabidae	*P. hypertrichosis*	Valentine, 1931	Terrestrial
Arachnida	Acari	Rhagidiidae	*Rhagidia varia*[a]	Zacharda, 1985	Terrestrial
Arachnida	Pseudoscorpionida	Chthoniidae	*Kleptochthonius henroti*	(Vachon, 1952)	Terrestrial

Note: *Pseudotremia fulgida* was previously reported from the study area (Loomis, 1943) but was not collected during the present study.
[a] Not previously recorded from study area.

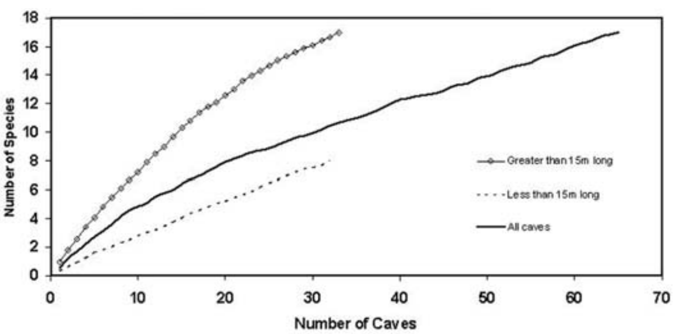

Figure 2. Rarefaction curves for number of caves versus number of species, for all caves (*n* = 65), caves less than or equal to 15 m (*n* = 32), and caves greater than 15 m (*n* = 33). Curves generated using EstimateS with the patchiness parameter set to 0.8 as recommended in Gotelli and Colwell (2001).

DISCUSSION

Prior to this study, knowledge of species richness from caves in this ca. 20 km² area was based on sampling of 9 caves (Holsinger *et al*. 1976; Fong & Culver 1994). In most of these collections, techniques other than hand sampling were not used (Holsinger *et al*. 1976). There are striking omissions from the previous faunal list, such as cave snails and flatworms, most likely due to a lack of an adequate census in the area (Fong & Culver 1994). There were 10 species known prior to our study.

Our study also had omissions. In spite of extensive hand collections and trapping, no spiders were recovered during our study. As a result of our efforts, the number of caves sampled in this area increased from nine to 65, the number of species recorded from this area increased from 10 to 18 (Table 1). When looking only at the nine caves that were resampled in the current investigation, seven species records were confirmed, and eight new localities were added for species previously reported from the study area. New taxonomic groups were also collected from these nine caves, including planarians, diplurans, collembola, millipedes and mites. As a result of this study, there were 93 new records of species, including eight new species, added to this roughly 20 km² area.

The eight species new to the study area are all known from West Virginia. Among the most notable species that we collected was the undescribed dipluran, *Eumesocampa* sp., which has only been collected from one other cave (Steeles Cave, Monroe County, West Virginia). Recent attempts to recollect this species in Steeles Cave have not been successful (L. Ferguson, pers. comm., 2002).

The findings of this research showed that subterranean biodiversity for this area had been greatly underestimated. Clearly, by focusing solely on the minimal information known from nine of 148 caves, many species would be unreported and the distributions of others incompletely known.

Ideally, homogeneous sampling and intensive sampling are preferred; however, subterranean areas are difficult and expensive to sample and the risk of overcollecting is usually a concern. Therefore, it is necessary to know the minimal sample size needed to get an accurate estimation of species diversity for an area. Using the "simple greedy" algorithm of Csuti *et al*. (1997), we found that only a small number of caves need to be sampled in order to collect all known species in the study area.

Table 2. Estimates of total cave-limited species richness in the study area.

Item	Estimate
Number of Caves	65
Number of Singletons	21
Number of Doubletons	12
Observed Number of Species	18
Chao's S_2*	36.4 ±1.1
Burnham and Overton's S_4*	47.6

Table 3. Cumulative numbers of cave-limited species based on the "simple greedy" algorithm of Csuti *et al.* (1997) applied to those caves that need to be sampled in order to collect 95% of total species.

Cave	New sp.
Buckeye Creek Cave	8
Matt's Black Cave	3
Upper Buckeye Creek Cave	2
Rapps Cave	2
Nellie's Cave	1
Hannah Caverns, Raceway Pit, Sunnyday Pit, Trilium Cave, Seep Cave 2, Short Stuff Cave, Tin Cave, or Wake Robin Cave	1
Total Species Collected	17

Table 4. Cumulative numbers of cave-limited species based on the "simple greedy" algorithm of Csuti *et al.* (1997) applied to the largest seven caves.

Cave	New sp.
Buckeye Creek Cave	8
Matt's Black Cave	3
Upper Buckeye Creek Cave	2
Rapps Cave	2
Hannah Caverns	1
McFerrin Water (Spur) Cave	0
Spencer Cave	0
Total Species Collected	16

We found that seven caves were sufficient to capture 95% of the known species (Table 3) although *a prioi* knowledge of which seven caves to sample is lacking. However, using cave length as a surrogate for species richness gives nearly the same results. By examining only the largest seven caves, 89% of the species were collected. This finding has conservation implications, as many of the species (including many of the rare species) could be protected by protecting the largest caves. Izquierdo *et al*. (2001), in a conservation application of Csuti *et al*.'s greedy algorithm, proposed that in order to maximize the number of species protected in a limited number of sites, conservation decisions should be focused on the cave with the most species, followed by the cave with the most species different from the first cave, and so on. If conservation decisions in the study area were indeed based according to this standard, then conservation priority would be given to the cave with the most species, in this case Buckeye Creek Cave. The next cave of concern would be the cave with the largest number of species different from the first, in this case, Matt's Black Cave. Here, following the guidelines of Izquierdo *et al*. and their application of the greedy algorithm, it would only take seven caves to protect 95% of the species. With this approach, many of the largest caves (and the species therein) would be protected. Protecting the largest caves does result in the protection of the greatest biodiversity, and the species accumulated in the larger caves represent most of the species found in the smaller caves.

Rarefaction curves generated from our data did not reach an asymptote, and the curve rose more steeply for larger caves than for smaller caves (Fig. 2) because species accumulated more quickly in larger caves. When no new taxa are added, an asymptote should, in principle, be reached (Gotelli & Colwell 2001). Due to the failure to reach an asymptote, total troglobitic and stygobitic species richness was estimated using equations provided by Colwell and Coddington (1994). Using Chao's estimate, S_2*, total species richness was 36 species, and it was 48 using the second-order jackknife estimate, S_4* (Table 2). Colwell and Coddington point out that in practice, the upper bound of the estimate for S_4* is approximately twice the observed number, i.e., 36, and the upper estimate for S_2* is approximately half the square of the observed number, i.e., 81. This in turn suggests that S_4* estimate is unreliable. If we use the S_2* value of 36 as the best estimate of the number of species, we have found only half of the species.

How did nearly half of the species evade collection? Over 90% of sampled caves in West Virginia have at least one troglobitic species (Culver *et al*. 2004). Here, only 69% of the sampled caves (45 of 65 caves) had at least one troglobite/stygobite collected and approximately one-third of the caves sampled yielded no troglobites or stygobites at all. Repeated visits often are necessary to collect all of the species found in a single cave. In one Italian cave, for example, Fabio Stoch determined that it took six trips to collect all 12 stygobites present (quoted in Culver *et al*. in press). In the nine caves that had been previously examined in our study area, we did not confirm 13 previous species occurrence records. This result could reflect either inadequate sampling or extirpation of these populations. We also did not sample all known caves. An additional 8 caves were found too late to be included in the study, and at least some of the 75 localities in the WVSS database that were reported as having a possible entrance may actually represent cave, at least for the species involved, even if they are not enterable.

With an increased sampling size, the detection of rare species increases (Huston 1994). That accumulation curves did not reach an asymptote (Fig. 2) indicates that not all species were discovered. This could indicate heterogeneity within the samples (Culver *et al*. 2004), because caves that have a majority of the troglobites and stygobites are few, whereas caves with few or no troglobites or stygobites are numerous. This could reflect the rarity of cave-limited taxa and differences in observability among species. The need for repeated sampling is evident. We estimated that the true number of species in this area is 36, twice the number of species collected in this study.

Summary

Although our data set is incomplete, it appears that (1) Previous estimates of richness for this 20 km² area were quite low and increasing the number of caves sampled from nine to 65 increased the number of species from 10 to 18; (2) Only a small number of the caves need to be sampled in order to collect all of the species observed; and (3) Based on rarefaction curves and mathematical estimations, half of the species from the study area were not collected despite this effort of intensive sampling. This study advances our understanding of cave-limited species, by providing insights into the richness and distribution of stygobites and troglobites, and assessing the efficacy and accuracy of current methods of quantifying subterranean biodiversity.

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