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Investigation of Sample Size Estimation for Measuring Quantitative Characteristics in DUS Testing of Shiitake Mushrooms

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Abstract: The sampling technique is commonly used in research investigations to more accurately estimate data with greater precision, at a lower cost and in less time. In plant DUS (distinctness, uniformity, and stability) testing, many quantitative characteristic data usually need to be obtained through individual measurements. However, there is currently no scientific method for determining the appropriate sampling size. The minimum number of testing samples for DUS testing was calculated based on the theory of sample size in descriptive studies and was validated through simple random sampling. The results show that the quantitative characteristics for the edible mushroom Shiitake (*Lentinula edodes*) in DUS testing were uniform. The calculated results show that 10 fruiting bodies for a single measurement were sufficient. Furthermore, the outcomes of the random sampling revealed that the mean of 10 samples did not significantly differ from the mean of all data. When the sample size exceeded 10, Cohen's kappa statistic suggested that the conclusion of distinctness was very close to the near-perfect agreement. Reducing the number of samples did not affect the uniform assessment. This study suggests that the theory of sample size in descriptive studies could be applied to calculate the minimum sample size in DUS testing, and for Shiitake DUS testing, measuring 10 fruiting bodies was sufficient.

Keywords: sample size; quantitative characteristics; DUS

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1. Introduction

In statistics, information is often inferred about a population by studying a finite number of individuals from that population, which is known as sampling. It is assumed that the characteristics of the sample are representative of the overall population. Simple random sampling is a natural starting point in a discussion of sampling because it is the simplest form of random sampling and serves as the foundation for many other random sampling methods [1]. Subsequently, the problem of sample size determination also became a common concern in the theory and practice of sampling surveys, which originated from the dilemma of sample size determination [2]. Correctly determining sample size involves a careful balancing act. Finding an appropriate sample size demands a clear understanding of the level of detail you wish to see in your data. A small sample size may result in a large estimator variance, reducing the statistical inference's credibility and increasing the probability of making errors during hypothesis testing [3]. If the sample size is too

small, even a well-conducted study might fail to accurately detect important effects or associations [4]. On the other hand, a large sample size can potentially enhance the precision of estimates, leading to a narrower margin of error, but also be wasteful in terms of money and resources, prolonging survey cycles and complicating studies. Therefore, determining the right sample size is an essential factor in any scientific research [5]. The sample size is the beating heart of any research project. A correct sample size will give life to your data making your findings robust, reliable, and believable.

Lentinula edodes (Berk.) Pegler, belonging to Basidiomycetes, Agaricales, Tricholomycetes, and Omphalotaceae [6], also known as Shiitake mushroom or Xianggu mushroom, is native to China, Japan, and other Far East Asian countries [7]. *L. edodes* is rich in minerals, vitamins, essential amino acids, and lentinan [8]. However, concerning the nutritional value of edible fungi, it should be considered that fresh fruiting bodies contain mainly water and only about 10% dry matter. For 100 g of fresh Shiitake, 92.6 g water, 4.3 g carbohydrates, 1.9 g proteins, 0.3 g lipids, 0.7 g ballast material, and 0.5 g minerals have been measured [9]. It is important to note that regarding its protein content, the data are too high because the protein amount was calculated on the nitrogen content measured with the Kjeldahl method, which includes also N of the chitin present in the hyphal cell walls [9]. Shiitake mushrooms are also well known for their anticancer, antitumor, antifatigue, antibacterial, antiviral and antioxidant activities [10]. Shiitake's popularity is increasing day by day due to its high nutritional value, unique flavor, taste, and enticing aroma [11]. The fruiting bodies are also considered as a fair substitute for meat, and their nutritional value is comparable to many vegetables. They have multiple nutrients which offer several nutritional and health benefits to mankind [12]. Moreover, *L. edodes* could prevent environmental impacts caused by the accumulation of forest and agricultural waste because it secretes hydrolytic and oxidative enzymes responsible for the degradation of organic substrates [13]. Shiitake mushrooms have been renowned in East Asian countries as a food and medicine for thousands of years. The cultivation of this mushroom has been practiced for thousands of years with its cultivation originating in China during the Sung Dynasty (960–1127) [14]. Wild Shiitake, on the other hand, has been harvested for at least 1800 years. The onset of the 20th century marked the dissemination of Shiitake mushrooms to the Western world [15]. Currently, the Shiitake mushroom is becoming popular in nutritional and medicinal products throughout Asia, Europe, and North America and is the second largest production quantity of edible fungi in the world after *Agaricus bisporus* [16]. Presently, China dominates over 95% of the total Shiitake mushroom production worldwide, which is approximately 7.6 billion kg [17]. *Lentinula edodes* holds the largest production scale in China's industry [18]. With its increasing popularity, Shiitake has become the leading contributor to worldwide mushroom production. The mushroom has the potential to be a crucial component in future food supplies and in new dimensions of sustainable agriculture and forestry.

The used or selected mushroom strain is the most important basic production material in mushroom production and plays a vital role in determining the yield and quality composition of mushrooms [19]. The development of superior cultivated strains is crucial for the sustainable growth of the modern mushroom industry [20]. China has developed over 100 commercial Shiitake strains [21]. In recent years, there has been an increasing emphasis on plant variety protection. To be eligible for protection under plant breeder's rights (PBRs), a plant variety must meet the distinctness, uniformity, and stability (DUS) requirements [22]. DUS testing plays a critical role in determining if a new variety differs from all existing varieties within the same species (distinctness). It also assesses if its characteristics used to establish distinctness are uniformly expressed (uniformity) and if these characteristics remain stable over subsequent generations (stability) [23]. Quantitative character is an important aspect of describing varieties in DUS testing. However, the investigation of the quantitative character demands a lot of manpower, so how to minimize manpower while maintaining the accuracy of the quantitative character has become an important topic for DUS testers. This is the main purpose of this paper.

Statistical methods are commonly used to assess the distinctness and uniformity of measured quantitative characteristics when the data from the growing trial for a variety are subject to variation. The combined over-years criteria for distinctness (COYD) is an appropriate method for evaluating the distinctness of varieties when the quantitative characteristic is observed for at least two years or growing cycles conducted at one location [24]. The combined over-years uniformity criterion (COYU) is used to assess the uniformity of a variety relative to comparable varieties based on the standard deviation from trials conducted over several years.

To determine the appropriate sample size, many methods can be utilized, such as adopting a sample size from a similar study, looking up a table, using a sample size calculator, or employing a formula [25]. Many books on statistics have tables that can be used to calculate sample size, and almost all statistical computer programs also yield a sample size when the power, significance level, and size of difference to be detected are entered [26]. Twisk [27] introduced several sample size calculation formulas. The document contains the standard sample size calculation formula for both continuous and dichotomous outcomes as well as the adjustments for either clustering (i.e., for a cluster randomized controlled trial) or designs with more than one follow-up measurements. There is no reliable and successful method for determining sample size based on a given confidence level and margin of error without knowing the population standard deviation for describing variety.

Currently, both China's guidelines and the UPOV test guidelines for Shiitake mushroom testing require that all observations on single fruiting bodies should be made on 60 fruiting bodies or parts taken from each of the 60 fruiting bodies [28,29]. This means that in DUS testing, each strain needs to measure 300 data points on fruiting bodies; it is labor-intensive and leads to a higher error rate. In our experiment, we conducted a significance analysis for 60 data points, aiming at 28 testing samples and five quantitative characteristics of fruiting bodies, listed in China's testing guidelines and the UPOV test guidelines for Shiitake mushrooms. The minimum number of samples for DUS testing was calculated based on the theory of sample size in descriptive studies and confirmed through simple random sampling.

2. Material and Methods

2.1. Time of the Test and Station

Data were collected from June 2019 to May 2020 (year 1) and from June 2020 to May 2021 (year 2). Cultivation tests were conducted at the Institute of Edible Fungi, Shanghai Academy of Agricultural Sciences (Shanghai, China).

2.2. Materials and Substrate

A total of 28 Shiitake varieties were used as testing samples. All strains were preserved at the institute mentioned above. The medium for stock culture was potato dextrose agar (PDA, Merck, Darmstadt, Germany). The composition of PDA included potato infusion (200 g), dextrose (20 g), agar (20 g), and distilled water (1000 mL). The cultivation substrate for the mother spawn and culture spawn consisted of sawdust (79%), wheat bran (20%), and gypsum (1%), with a moisture content ranging from 55% to 60%.

2.3. Shiitake Incubating Method

The sawdust bag log technology, which is widely spread among mushroom growers in the Orient region, was used to cultivate Shiitake mushrooms according the technical code of practice for the intensive production of Xianggu (*Lentinula edodes*) artificial bed-log [30]. The plastic mushroom growing bag size was 150 mm × 550 mm × 0.05 mm, and the cultivation substrate (2 ± 0.1 kg per bag) was filled into the bags using a filling machine. After filling, the length of the mushroom sticks was 450~500 mm. The bags were then autoclaved at 121 °C for 3 h and cooled before inoculation with the mother spawn. Each strain was inoculated into 120 bags with 3 drills per bag. The inoculated bags were

incubated at 22 °C to 25 °C with 60% to 70% relative humidity and CO_2 concentration below 2500 ppm for 30~40 days in dark conditions. Ventilation was improved by puncturing the mycelium when the diameter of growing hyphae reached approximately 10 cm. When the mycelium had fully covered the substrate, we exposed the bags to fluorescent light ranging from 50 Lux to 300 Lux for 4 h per day and reduced the CO_2 concentration to below 2000 ppm. After the mushroom sticks had turned brown, the plastic bags were removed, and the sticks were transferred to an outdoor cultivation area for primordial initiation. The environmental temperature ranged from 14 ± 2 °C (night) to 22 ± 3 °C (day) with a relative air humidity of 85% to 90% and 500 Lux to 1500 Lux of natural sunlight, and CO_2 concentration was between 1000 and 1500 ppm. Once primordia formed, the temperature was decreased, keeping 10 °C to 18 °C with a relative air humidity of 60% to 80%, and CO_2 concentration was controlled below 1000 ppm for developing fruiting bodies.

Each variety was divided into two blocks, and 60 sticks were contained in each replication. All data were collected from the second flush of fruiting bodies.

2.4. Measurement of Quantitative Characteristics

Five quantitative characteristics, pileus height (ch1), stipe length (ch2), stipe diameter (ch3), ratio of pileus diameter to stipe length (ch4), and ratio of pileus diameter to stipe diameter (ch5), were measured for each mushroom strain, and 60 data points were collected from 60 fruiting bodies for each characteristic.

2.5. Data Analysis

Descriptive statistics were performed for each trait using EXCEL 2019, and the coefficient of variation (CV) of each trait was calculated by dividing the standard deviation by the mean. Additionally, the Shapiro—Wilk test was performed using XLSTAT 2019.

The sample size was calculated according to the sample size in descriptive studies [31]. The sampling estimation error allowed by the trial population parameters was determined as the extreme quarter (quartile moment) based on practical experience. The needed sample size was calculated as follows:

$$n = (\frac{SD}{SE})^2$$

SE is the sampling estimation error divided by 2.56 (for the 95% confidence interval), and SD is the standard deviation.

A random sampling program was developed using Python 3.9. Different number of samples, 5, 10, 20, 30, 40, and 50 samples, were randomly selected from 60 data points three times. Subsequently, various multiple comparisons were conducted among the different samples.

According to TGP/8 [32], the least significant difference (LSD_p) and the maximum allowable standard deviation (the uniformity criterion, UC_p) were calculated as follows:

$$LSD_p = t_p \times \sqrt{2 \times SE(\bar{x})}$$

where $SE(\bar{x})$ is the standard error of a variety's over-year mean and t_p is the value in Student's *t* table appropriate for a two-tailed test with probability *p* and with degrees of freedom associated with the variety-by-years mean square. The probability level *p* is 0.01.

To assess the distinctness of a candidate, the difference in the means between the candidate and all other varieties was computed. In practice, a column of differences was calculated for each candidate. In this case, varieties with mean differences greater than or equal to LSD_p were regarded as distinct (marked D).

$$UC_p = SD_r + t_p \sqrt{V(\frac{1}{k} + \frac{1}{R \times k})}$$

where SD_r is the mean of adjusted log (SD_s + 1) for the comparable varieties, V is the variance of the adjusted log(SD_s + 1) after removing year effects, t_p is the one-tailed t value

for probability $p = 0.002$ with degrees of freedom as for V, k is the number of years, and R is the number of comparable varieties.

Within-plot standard deviations for each variety in each year were calculated by averaging the plot between-plant standard deviations, SD_j, over replicates:

$$SD_j = \sqrt{\frac{\sum_{i=1}^{n} (y_{ij} - y_j)^2}{(n-1)}}$$

$$SD = \frac{\sum_{j=1}^{r} SD_j}{r}$$

where y_{ij} is the observation on the i^{th} plant in the j^{th} plot, y_j is the mean of the observations from the j^{th} plot, n is the number of plants measured in each plot, and r is the number of replicates.

The trend value Tc for the candidate is given by:

$$T_c = \frac{(X_c - X_i)T_{i+1} + (X_{i+1} - X_c)T_i}{X_{i+1} - X_i}$$

$$\text{Adjusted log(SD + 1)} = \text{Log(SD + 1)} - T_c + \text{mean}$$

where mean is the average of log(SD + 1) of reference varieties.

Varieties with a mean adjusted log(SD + 1) less than or equal to UC_p were regarded as uniform.

3. Results

3.1. Statistical Description and Test of the Normal Distribution of the Measurement Data

All measured traits showed variation among 28 varieties, with the minimum value ranging from 0.37 to 0.66 of the maximum value. The coefficient of variation (CV) within the variety of traits was less than 20% in the two years (Table 1), indicating a high degree of uniformity within varieties and exhibiting sufficient variation between varieties that fulfilled the requirements for DUS testing [24]. However, the two-year data on pileus height and stipe diameter showed significant variations.

Table 1. Statistical description of 28 Shiitake varieties.

Year	Characteristic	Max	Min	Mean	Mean SD	CV/%
Year 1	Pileus height/mm	22.00	13.31	18.30	2.88	15.71
	Stipe length/mm	46.74	30.28	39.30	6.24	15.89
	Stipe diameter/mm	23.12	11.54	16.81	2.86	17.01
	Ratio of pileus diameter to stipe length	1.75	0.92	1.46	0.23	16.04
	Ratio of pileus diameter to stipe diameter	4.14	2.75	3.45	0.58	16.91
Year 2	Pileus height/mm	22.33	9.43	16.64	2.70	16.24
	Stipe length/mm	44.71	26.88	38.21	5.87	15.36
	Stipe diameter/mm	22.95	10.63	17.31	2.77	16.00
	Ratio of pileus diameter to stipe length	1.74	1.15	1.50	0.23	15.59
	Ratio of pileus diameter to stipe diameter	2.97	1.10	1.93	0.31	16.09

According to the Shapiro—Wilk test, a large portion of the data from 28 varieties over 2 years did not adhere to a normal distribution. Specifically, the measurement of stipe

length exhibited the lowest proportion of data that conformed to a normal distribution (Table 2).

Table 2. Shapiro—Wilk test results.

Characteristic	Year 1	Year 2
Pileus height	9/19	17/11
Stipe length	4/24	5/23
Stipe diameter	16/12	14/14
Ratio of pileus diameter to stipe length	9/19	15/13
Ratio of pileus diameter to stipe diameter	11/17	7/21

Note: The data in the table consist of a normally distributed variety number and a non-normally distributed variety number.

3.2. Minimum Sample Size Calculation Results

The minimum sample size of five quantitative traits of Shiitake mushrooms was determined using the sampling sample size calculation formula, and the results are presented in Figure 1. The results indicate that the minimum sample size varied among different quantitative traits, "ratio of pileus diameter to stipe length" (ch4) and "ratio of pileus diameter to stipe diameter" (ch5) required at least 9 samples, while "pileus height" (ch1), "stipe length" (ch2), and "stipe diameter" (ch3) needed 10 samples. It can be inferred that describing Shiitake mushroom varieties requires measuring 10 fruiting bodies.

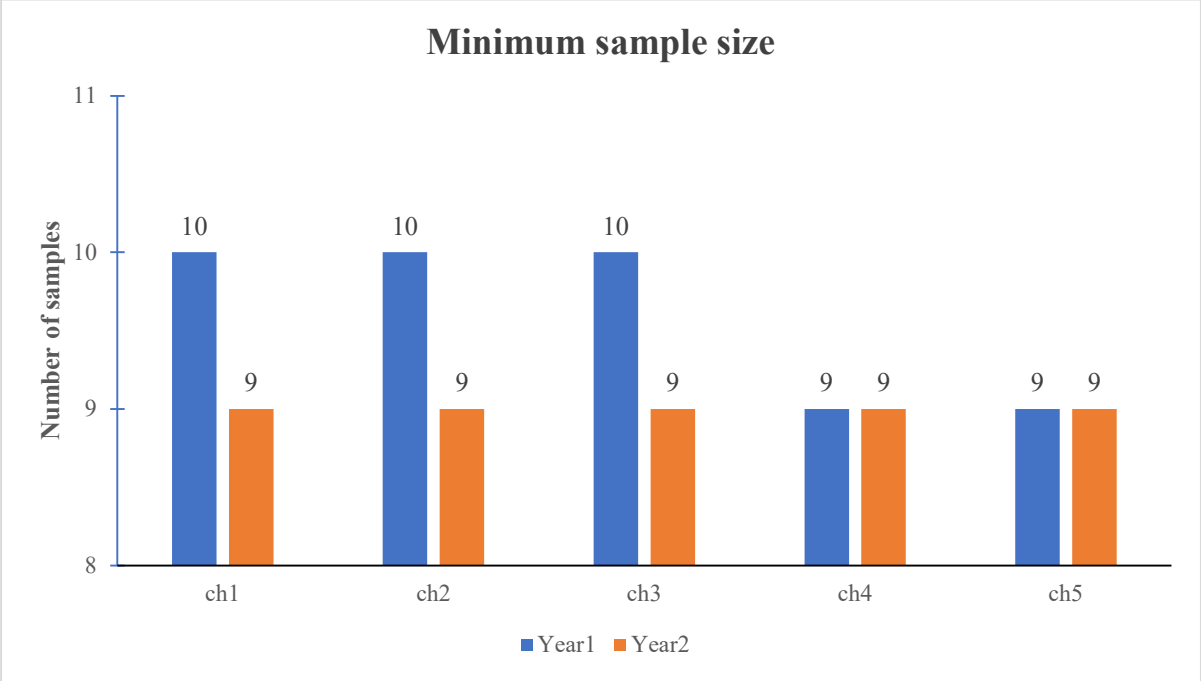
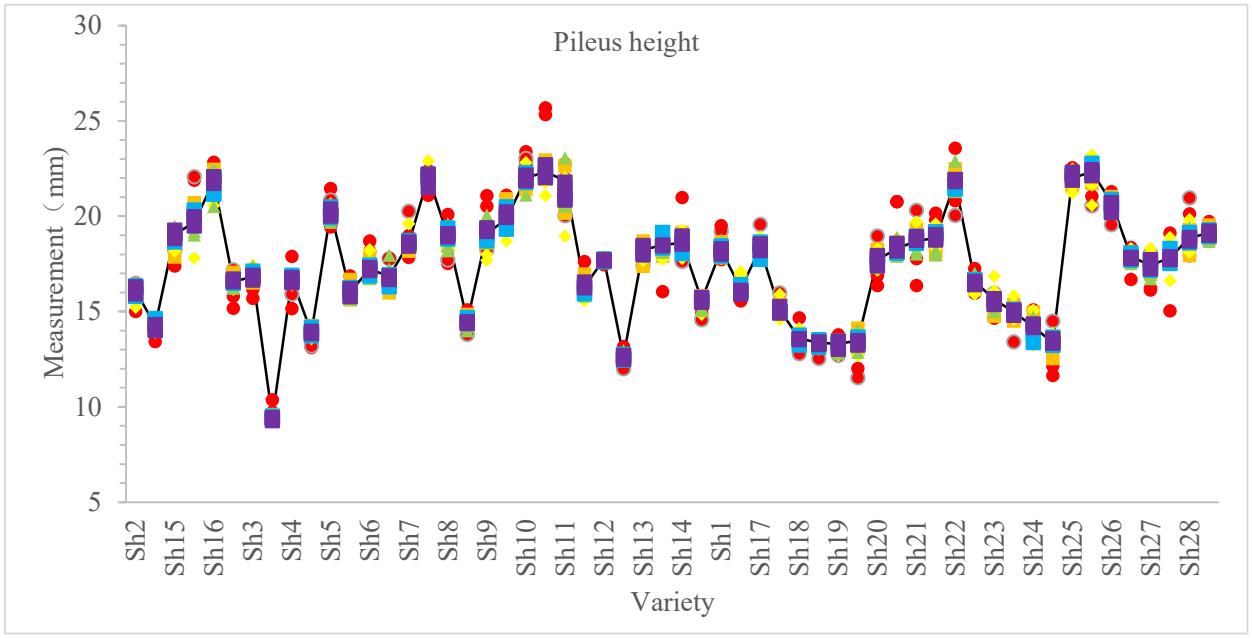


Figure 1. Calculated minimum sample size of 5 quantitative characteristics. ch1: pileus height, ch2: stipe length, ch3: stipe diameter, ch4: ratio of pileus diameter to stipe length, and ch5: ratio of pileus diameter to stipe diameter.

3.3. Random Sampling Results

Various numbers of samples were randomly selected from a pool of 60 data points, and the average of each selection was calculated. The results show that the five traits displayed a similar tendency, with a slight difference between the average and the original values. Furthermore, it was observed that as the sample size increased, the average differences decreased (Figure 2). Additionally, it was found that the average of random sampling was different even when the sample size was the same, with the largest difference observed

between the original average and the results of five samples. This indicated that to minimize the deviation of the data, a minimum of 10 samples was needed.



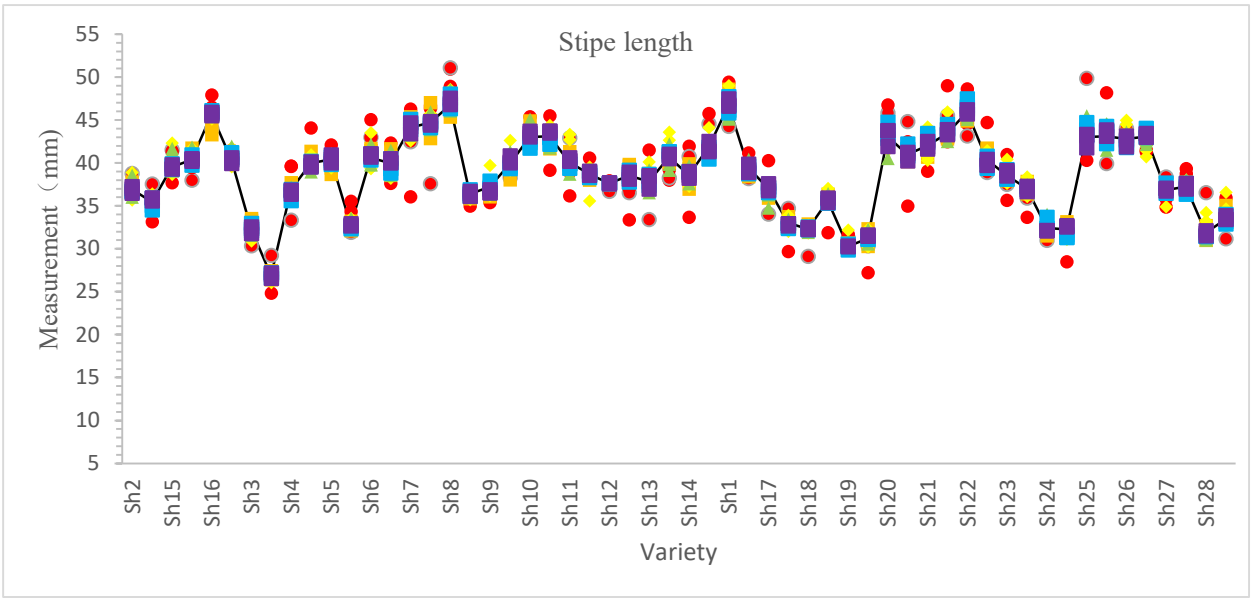


Figure 2. *Cont.*

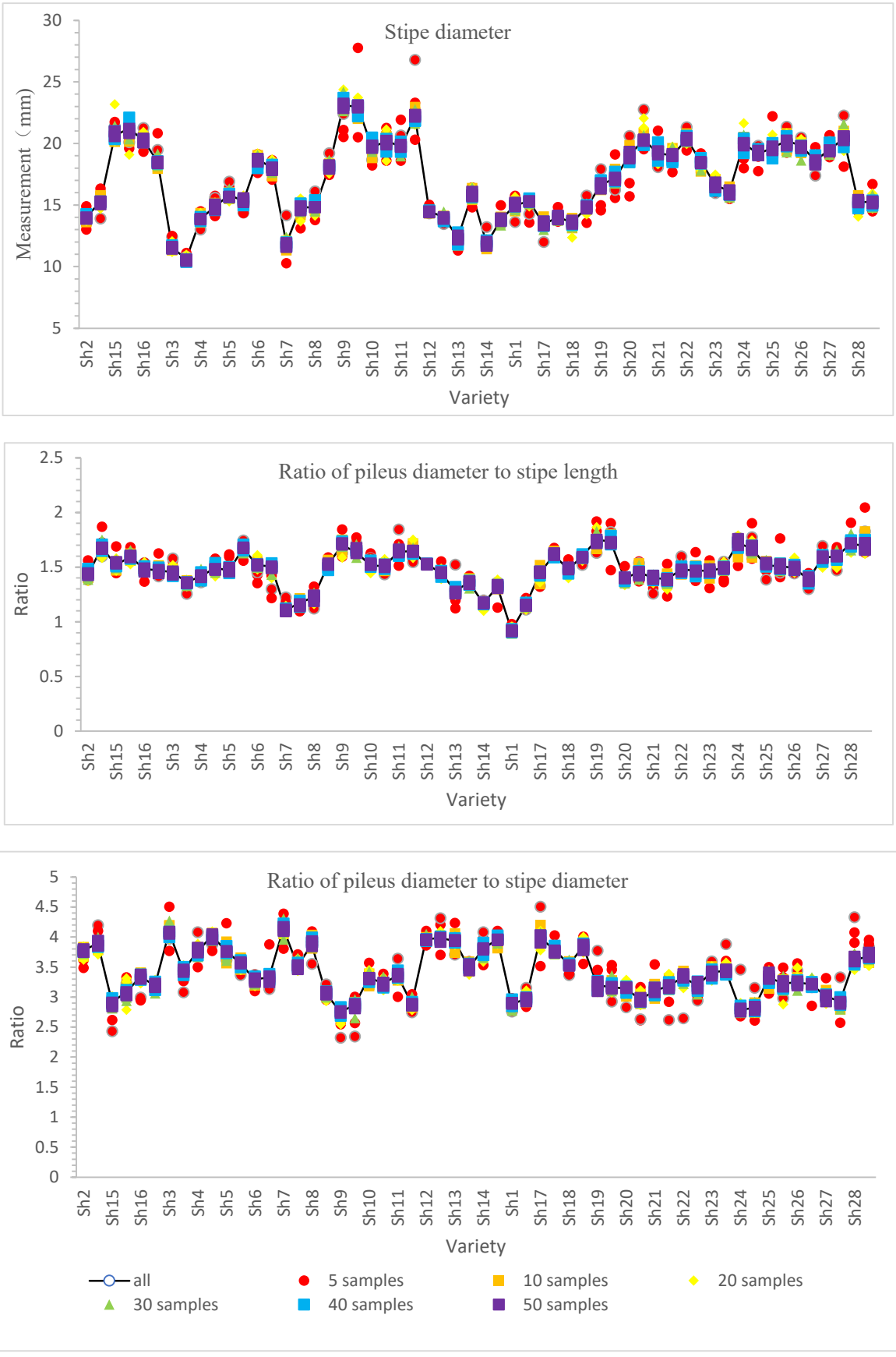


Figure 2. The mean of the different sample sizes of the 28 varieties. Note: Each variety has 2 values: the first is the mean of year 1, and the second is the mean of year 2.

Using multiple comparisons for each trait, we found that almost all traits of the 28 varieties did not show any significant differences between different sample size results. The exception was the ratio of pileus diameter to stipe diameter of one variety (sh22), where the mean value of 30 samples was the highest, and one mean of 5 samples was the smallest, and there was no significant difference between other groups, as detailed in Table 3. Reducing the number of observations from 60 to 10 will result in an error of 6.77%.

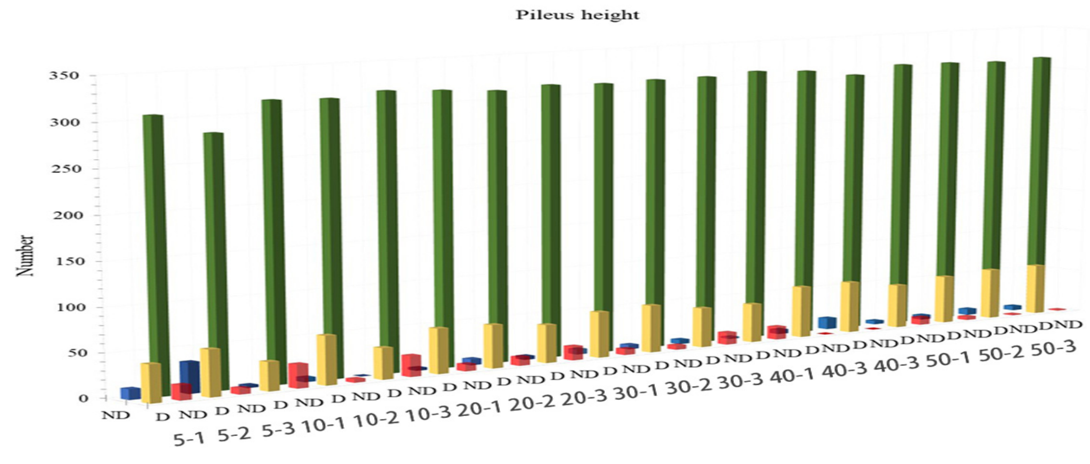
Table 3. Multiple comparison results of the ratio of pileus diameter to stipe diameter in variety sh22.

Sample Size	Mean	Label
all	3.337	a,b
50-3	3.345	a,b
50-2	3.329	a,b
50-1	3.387	a,b
40-3	3.310	a,b
40-2	3.308	a,b
40-1	3.278	a,b
30-3	3.363	a,b
30-2	3.373	a,b
30-1	3.441	a
20-3	3.155	a,b
20-2	3.403	a,b
20-1	3.357	a,b
10-3	3.361	a,b
10-2	3.111	a,b
10-1	3.286	a,b
05-3	3.402	a,b
05-2	2.652	b
05-1	3.404	a,b

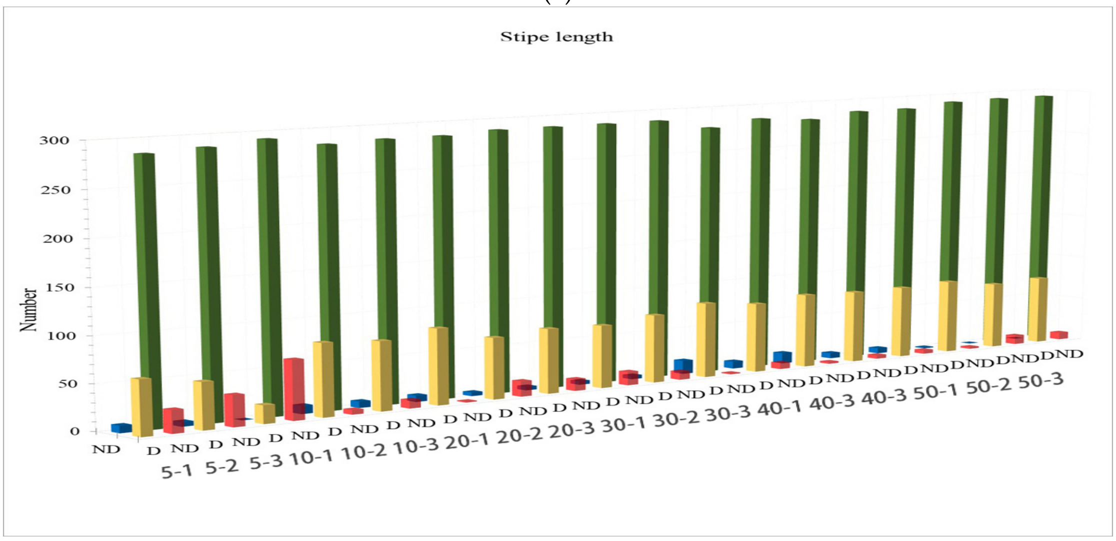
Note: All: 60 samples, 50-3: the third random sampling of 50 samples, and other codes proceed according to this rule. For all groups with the same letter, the difference between the means is not statistically significant at the 0.05 level.

3.4. Effect of Sample Size on Combined Over-Years Criteria for Distinctness

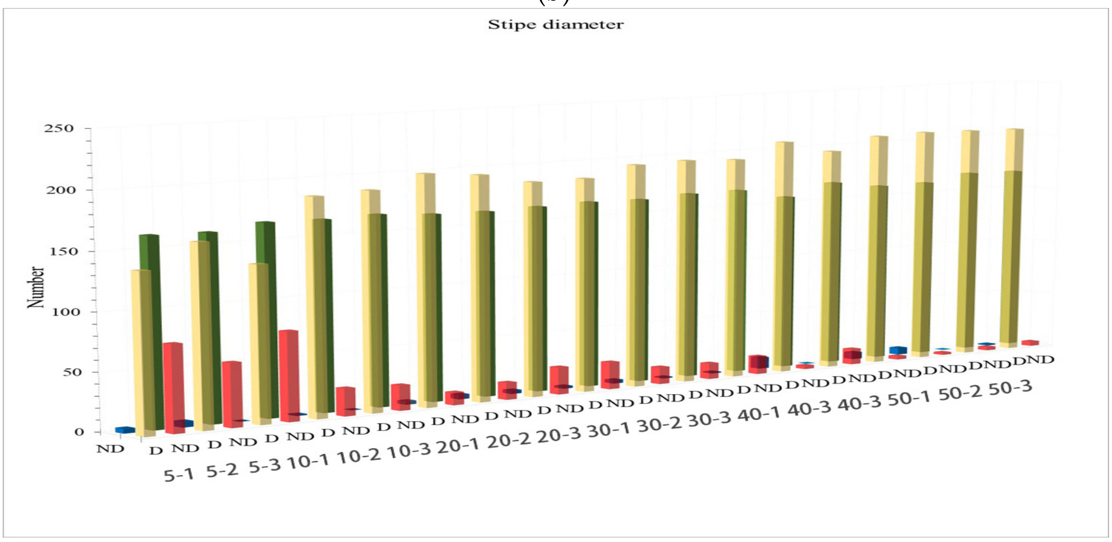
The 28 varieties were compared pairwise, resulting in 378 pairs. The conclusion of whether there is a difference between varieties varied in different sample sizes. Assuming the conclusions based on all the samples are correct, two errors were identified in each random sampling test. The first error, known as type I error occurred when different varieties were assessed as having no difference. The second error, referred to as a type II error, happened when pairs of varieties without differences were mistakenly judged as different. In most traits, except stipe diameter, the proportion of non-different (ND) was much higher than the proportion of different (D), resulting in a higher type I error compared to type II error. With an increase in sample size, the proportion of misjudgments decreased significantly. When the sample size is small, such as five samples, the type I error of some traits (ch4 and ch5) may be as high as 50% (Figure 3). The Cohen's kappa coefficients among five samples and all samples had the following ranges: 0.645~0.703 (ch1), 0.323~0.730 (ch2), 0.595~0.688 (ch3), 0.435~0.579 (ch4), and 0.351~0.469 (ch5). These coefficients indicated fair to substantial agreement.



(**a**)

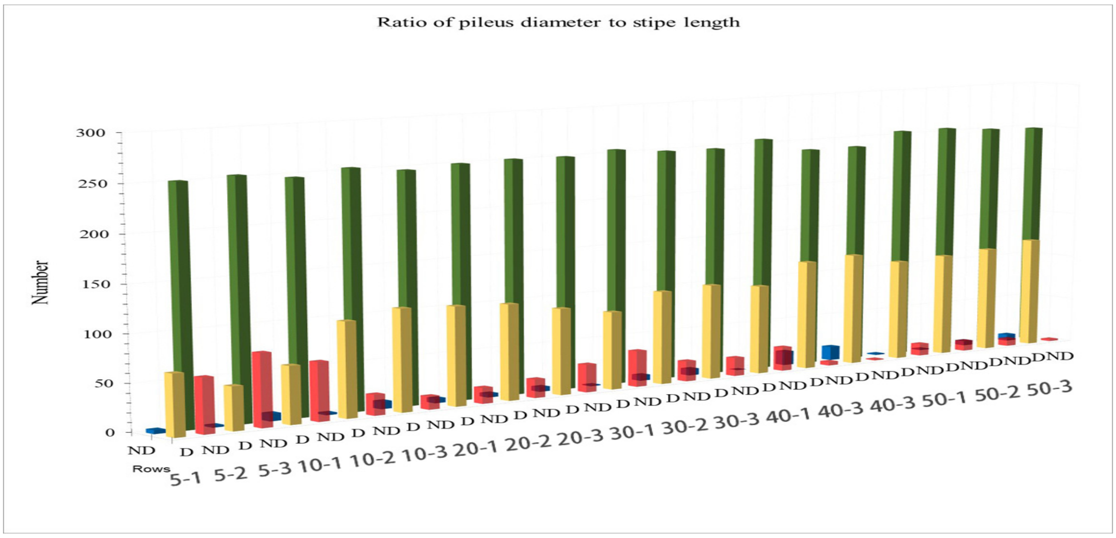


(**b**)

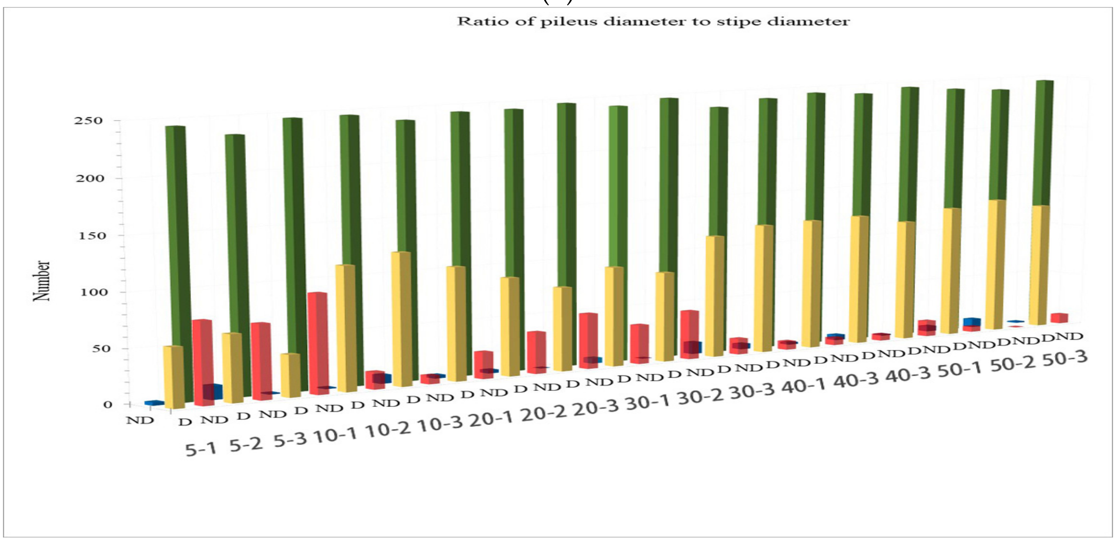


(**c**)

Figure 3. *Cont*.



(**d**)



(**e**)

Figure 3. Determination results by combined over-years criteria for distinctness for different sample sizes. (**a**): pileus height; (**b**): stipe length; (**c**): stipe diameter; (**d**): ratio of pileus diameter to stipe length; and (**e**): ratio of pileus diameter to stipe diameter. Type I error: red column; Type II error: blue column; ND: green column; and D: yellow column.

3.5. Effect of Sample Size on Combined Over-Years Uniformity Criterion

The UC_p value varied across different sample sizes (Table 4). The maximum UC_p values for most traits were obtained from 5 samples, except for stipe diameter, for which the largest UC_p was from 60 samples.

For different sample sizes, the COYU calculation results show that the adjusted log (SD + 1) values of the 5 test varieties were all less than UC_p, indicating that all varieties were uniform. Different sample sizes affected the UC_p and adjusted log(SD + 1) of each variety; this was also found when the sample size was the same. The difference in UC_p and adjusted log(SD + 1) was primarily due to variations in the mean and standard deviation, and it had no obvious effect on the judgment results.

Table 4. Adjusted log(SD + 1) of five test varieties with different sample sizes.

Characteristic		All	05-1	05-2	05-3	10-1	10-2	10-3	20-1	20-2	20-3	30-1	30-2	30-3	40-1	40-2	40-3	50-1	50-2	50-3
Pileus height	UC_p	1.74	1.78	2.25	1.88	2.14	2.23	2.15	2.07	1.77	1.83	1.97	1.98	1.86	1.94	1.95	1.94	1.75	1.93	1.92
	Sh1	1.2	1.4	1.17	1.43	1.58	1.56	1.41	1.51	1.26	1.2	1.41	1.42	1.35	1.41	1.44	1.34	1.26	1.42	1.41
	Sh2	1.36	1.56	1.36	1.55	1.58	1.69	1.62	1.71	1.47	1.48	1.7	1.63	1.5	1.65	1.59	1.51	1.43	1.59	1.58
	Sh3	1.06	1.21	1.41	1.32	1.2	1.43	1.35	1.4	1.14	1.16	1.34	1.31	1.18	1.22	1.28	1.19	1.11	1.26	1.26
	Sh27	1.56	1.62	1.85	1.72	1.65	1.85	1.87	1.89	1.61	1.64	1.9	1.81	1.73	1.8	1.83	1.7	1.61	1.79	1.76
	Sh28	1.25	1.22	1.49	1.25	1.46	1.58	1.45	1.55	1.38	1.34	1.46	1.46	1.4	1.55	1.49	1.4	1.32	1.48	1.44
Stipe length	UC_p	2.47	2.62	2.77	3.13	2.6	2.71	2.82	2.67	2.62	2.64	2.64	2.67	2.67	2.62	2.51	2.65	2.62	2.62	2.63
	Sh1	1.95	2.25	1.85	2.58	1.82	2.14	2.09	2.04	2.03	2.16	2.03	2.04	2.08	2.02	1.96	2.2	2.11	2.11	2.08
	Sh2	2.01	1.85	2.16	2.23	1.91	2.17	2.48	1.99	2.14	2.03	2.2	2.03	2.15	2.11	1.96	2.2	2.08	2.12	2.13
	Sh3	1.79	1.66	1.77	1.86	1.97	1.92	2.16	2.07	1.87	1.99	1.93	1.92	1.94	1.99	1.81	2.01	1.9	1.91	1.98
	Sh27	2.19	2.15	2.2	2.22	2.3	2.31	2.45	1.96	2.48	2.22	2.26	2.35	2.26	2.21	2.23	2.36	2.31	2.25	2.34
	Sh28	2.07	2.14	2.32	2.41	2.11	2.02	2.39	1.9	2.11	2.11	2.27	2.31	2.17	2.19	2.1	2.27	2.23	2.2	2.21
Stipe diameter	UC_p	2.13	1.7	1.83	2.02	2.05	1.91	1.9	1.89	1.96	1.96	1.76	1.82	1.85	1.91	1.89	1.88	1.9	1.89	1.91
	Sh1	1.24	0.87	1.08	1.29	1.57	1.5	1.61	1.47	1.52	1.52	1.45	1.47	1.48	1.51	1.55	1.49	1.52	1.54	1.52
	Sh2	1.22	1.13	1.28	1.63	1.52	1.43	1.52	1.6	1.52	1.67	1.45	1.51	1.52	1.51	1.58	1.52	1.55	1.56	1.59
	Sh3	1.09	0.96	1.23	1.17	1.34	1.44	1.44	1.32	1.37	1.43	1.26	1.24	1.43	1.37	1.38	1.38	1.39	1.4	1.42
	Sh27	1.43	1.57	1.34	1.61	1.75	1.68	1.69	1.7	1.75	1.62	1.51	1.54	1.52	1.62	1.54	1.58	1.63	1.29	1.65
	Sh28	1.32	1.05	1.49	1.23	1.78	1.45	1.65	1.61	1.58	1.61	1.43	1.56	1.6	1.61	1.64	1.51	1.6	1.62	1.59
Ratio of pileus diameter to stipe length	UC_p	0.29	0.31	0.34	0.4	0.36	0.29	0.33	0.36	0.31	0.34	0.3	0.3	0.32	0.32	0.31	0.29	0.31	0.31	0.31
	Sh1	0.16	0.13	0.13	0.24	0.18	0.12	0.21	0.22	0.18	0.19	0.16	0.17	0.19	0.2	0.18	0.16	0.19	0.18	0.18
	Sh2	0.19	0.12	0.14	0.21	0.25	0.1	0.22	0.26	0.18	0.24	0.2	0.2	0.21	0.2	0.22	0.21	0.21	0.22	0.22
	Sh3	0.19	0.16	0.09	0.25	0.2	0.17	0.23	0.23	0.21	0.25	0.19	0.19	0.22	0.22	0.22	0.18	0.22	0.22	0.21
	Sh27	0.25	0.16	0.16	0.22	0.19	0.25	0.15	0.24	0.19	0.22	0.19	0.25	0.26	0.27	0.27	0.22	0.25	0.27	0.28
	Sh28	0.21	0.09	0.21	0.2	0.21	0.2	0.25	0.25	0.21	0.23	0.23	0.23	0.24	0.23	0.24	0.2	0.23	0.21	0.23
Ratio of pileus diameter to stipe diameter	UC_p	0.58	0.75	0.62	0.71	0.7	0.66	0.63	0.61	0.63	0.59	0.64	0.64	0.61	0.63	0.56	0.58	0.6	0.58	0.63
	Sh1	0.33	0.39	0.39	0.35	0.43	0.42	0.22	0.28	0.41	0.36	0.42	0.33	0.37	0.38	0.31	0.33	0.34	0.34	0.38
	Sh2	0.45	0.62	0.55	0.44	0.55	0.52	0.48	0.49	0.42	0.4	0.47	0.43	0.45	0.46	0.43	0.43	0.49	0.46	0.49
	Sh3	0.45	0.33	0.3	0.46	0.4	0.45	0.81	0.44	0.46	0.4	0.48	0.44	0.43	0.48	0.46	0.42	0.48	0.43	0.48
	Sh27	0.45	0.33	0.29	0.56	0.51	0.47	0.51	0.42	0.5	0.47	0.5	0.49	0.45	0.53	0.45	0.46	0.48	0.43	0.46
	Sh28	0.49	0.22	0.43	0.5	0.53	0.45	0.47	0.55	0.53	0.51	0.56	0.52	0.49	0.54	0.43	0.45	0.52	0.51	0.53

4. Discussion

The results of this study on Shiitake mushrooms indicate that the frequency distribution of all surveyed agronomic traits was in the form of continuous variation, which is consistent with the research of Li et al. [33]. The coefficient of variation (CV) for all characteristics of fruiting bodies in Shiitake mushroom varieties was less than 20%. This suggests that the cultivated strains are consistent, which aligns with the findings of Chiu et al. [34]. This consistency may be a result of the high-intensity artificial selection of each agronomic trait during the breeding process or strict environmental control during cultivation. The characteristics of fruiting bodies are crucial for the commodity value of Shiitake mushrooms. The majority of customers tend to purchase Shiitake fruiting bodies with thick and meaty caps [35]. Therefore, these characteristics are also the focus of breeders. It is speculated that DUS testing does not need to measure many individuals. In many studies, the phenotype evaluation of Shiitake was measured using at least 10 fruiting bodies of each strain, such as Zheng et al. [36]. Our results show that 10 fruiting bodies were sufficient for the DUS testing of Shiitake.

The International Union for the Protection of New Varieties of Plants (UPOV) has developed DUS guidance, including a general introduction to DUS and the associated series of documents specifying test guideline procedures. In order to describe the varieties to be tested, the range of expression of each quantitative characteristic in the test guidelines is divided into a number of states, and a numerical 'Note' is attributed to each state [24]. The ratio of pileus diameter to stipe diameter of sh22 showed the highest difference, and the mean of 10 samples was 3.111, reducing 6.77%. However, this did not change the Note significantly, because the state interval for this characteristic is 0.4, which is twice the observed difference.

As we know, the statistics of samples are variable when sampling from the same population, and even if the sample size is the same each time, the statistical results will not be identical. There will always be a certain error, but the size of this error generally has a range. This was confirmed by the sampling results of different batches with the same sample size. Generally, if the error falls within a certain range, the sample statistics can be used to estimate the overall parameter; otherwise, they cannot be used as the valuation of the overall parameter. The greater the degree of variation in the sample, the larger the sampling estimation error, and the less reliable the estimation; the sampling estimation error is inversely proportional to the square root of the sample size. That is, the larger the sample, the smaller the sampling estimation error, and the more accurate the estimation; an estimation error that is too large may lead to incorrect conclusions. The sample size plays a crucial role not only in ensuring the scientific validity and precision of testing but also in minimizing the cost of testing. The size of the sample is very important for obtaining accurate, statistically significant results and successfully conducting your study. If your sample is too small, you may include a disproportionate number of individuals that are outliers and anomalies. These skew the results, preventing a fair representation of the entire population. If the sample size is too large, the whole study becomes complex, expensive, and time-consuming to run, although the results may be more accurate, the benefits do not outweigh the costs. The results show that a larger sample size tended to lead to a lower probability of type I errors and type II errors. The sample size should therefore be chosen to give an acceptably low level of errors. However, small increases in the sample size may not always be advantageous. DUS testing is crucial for determining if new plant varieties can be granted protection rights. Jin Wenlin et al. estimated the sample means and concluded that a sample size of 20~30 was more appropriate for the comprehensive examination of crop traits [37]. In this article, the minimum sample size was determined based on the analysis of five quantitative traits in Shiitake mushrooms. Both calculation and random sampling indicated that 10 fruiting bodies were the minimum for measuring Shiitake quantitative characteristics. The sample size has been significantly reduced, which can decrease the workload in practical work and the DUS testing cost.

The propagation of the Shiitake strains was primarily by vegetative means [7]. In self-pollinated and vegetatively propagated varieties, where all the individual plants of a variety are expected to be quite similar, it is possible to assess uniformity by the number of off-type plants. The acceptable number of off-types tolerated in a variety is typically determined by a fixed population standard and acceptance probability. With the same standard and varying sample sizes, the maximum number of off-types would be different. Hence, the determination of the sample size should consider the uniform standard of this crop. For the assessment of Shiitake uniformity, a population standard of 1% and an acceptance probability of at least 95% should be applied [29]. In the case of a sample size of 60 fruiting bodies, two off-types are allowed and one off-type for 10 fruiting bodies. In order to avoid misjudging uniformity because of a decreasing sample size, the sampling capacity for group observations can be different from that of individual observations.

The number of samples for single measurement characteristics is one of the important contents of the test guidelines. It is affected by the breeding scheme, the degree of variety uniformity, and the method of assessing distinctness [32]. In the 338 UPOV crop-specific test guidelines, the stipulation is for 3~90 plants or parts of plants to be examined. For example, the blue honeysuckle (*Lonicera caerulea* L.) requires only three plants [38], while oyster mushroom examinations should be made on 90 fruiting bodies [39]. Among them, 40 test guidelines recommend that unless otherwise indicated, for the purpose of distinctness, all observations on individual plants should be made on 60 plants or parts taken from each of the 60 plants and any other observations made on all plants in the test, disregarding any off-type plants, including Shiitake mushrooms [29]. Of all the guidelines, there were only three testing guidelines for edible fungus, namely, oyster mushroom (*Pleurotus ostreatus* (Jacq.) P. Kumm), Shiitake mushroom, and agaricus (*Agaricus bisporus* (Lange.) Sing.), and only 30 fruiting bodies of agaricus were measured in the DUS test. The results of this study indicate that measuring 10 fruiting bodies was adequate for Shiitake mushrooms, which would greatly reduce the workload. China has published 14 guidelines for testing edible fungus, which require 30–120 fruiting bodies. If the sample size can be reduced to 20 or even fewer through calculation, it can be predicted that the measurement of labor and planting costs will be greatly saved.

Factories try to obtain high-quality mushrooms over short times (e.g., the 1st and 2nd flushes), while farmers often look for further benefits and harvest until the 4th flush [40]. The yield of each flush varies among different varieties; the initial two flushes account for more than 80% of the total production [41], and the first flush yield of some varieties is notably low. The agronomic traits of the fruiting body decrease with each increasing flush, except for the length of the stipe, which was the shortest in the first flush and the longest in the second flush [42]. Although harvesting the second flush will take more time, for the full expression of a variety of traits, the second-flush fruiting bodies were measured for this study.

5. Conclusions

In this study, the theory of sample size in descriptive studies was used to calculate the minimum sample size for five quantitative traits in Shiitake mushroom DUS testing. This study will significantly reduce the test workload by decreasing the sample size from 60 to 10. Moreover, the sample size does not have a significant impact on the average value, distinctness, and uniformity determination. To our knowledge, this work is the first to apply the theory of sample size in descriptive studies to the sample size calculation of edible fungi DUS testing. This can provide a scientific basis for the development of similar specialized DUS test guidelines.

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