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Testing guidelines for distinctness, uniformity and stability in *Oxalis*

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Research Article

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Abstract

The genus *Oxalis* contains important species worldwide with great economic and edible values. However, the testing guidelines have not been furbished, especially in China. *Oxalis* germplasm, including 60 species and 125 varieties, was collected from home and abroad and extensive field trials and phenotypic observations were conducted along with quantitative taxonomy, observations, correlation analysis and cluster analysis. Under the test guidelines of the International Union for the Protection of New Plant Varieties (UPOV), general guidelines for testing for distinctness, uniformity, stability (DUS) of new plant varieties, and Japanese test guidelines for DUS of *Oxalis*, 96 test characteristics (38 qualitative characteristics, 28 quantitative characteristics and 30 pseudo-qualitative characteristics) were determined as DUS test characteristics of *Oxalis*. Each test characteristic was scientifically graded and accurately described, and standard varieties and characteristic diagrams were provided for some characteristics. The guidelines for testing DUS of new plant varieties of *Oxalis* provide a standard for examining and testing new varieties of plants.

Introduction

Oxalis is an important genus of Oxalidaceae family and includes annual or perennial herbaceous plants. The centre of diversity of Oxalidaceae is in South America and South Africa and *Oxalis* contains more than 800 species worldwide (Burger, 1992). There are 8–9 species of Chinese origin and naturalization (including subspecies grade) (Liu *et al*., 2008). The plants of this genus can be divided into three main types according to their functions: ornamental, medicinal and edible. Among them, the ornamental *Oxalis* has many advantages, such as easy cultivation and propagation, strong adaptability, good population effect, long flowering period, rich flower colour, diverse leaf shapes and variegated leaf colours. Many varieties have gained year-round appreciation due to their wide use in landscaping and as potted ornamental plants. Moreover, they are also popular material for scientific research (Ágoston, 2017; Sarkar *et al*., 2020).

New plant variety protection is a form of intellectual property protection that protects the plant itself and the legitimate rights and interests of breeders (Correa *et al*., 2015). To date, the international situation for protection has improved and the international regulations for protection are well established. Understanding the new plant variety protection system and using relevant laws and regulations to protect the legitimate rights and interests of breeders are the prerequisite and foundations for implementing new plant variety protection. The regulations on plant variety protection in China stipulate that granting new plant variety rights must meet the requirements of DUS. Therefore, it is necessary to develop DUS test guidelines for new varieties of the Chinese *Oxalis*. In recent years, Chinese potted flower industry has been booming, which has greatly promoted the development of flower breeding in China (Zhang *et al*., 2014). With good ornamental value and application prospects, *Oxalis* is rich in germplasm resources and one of the most commonly grown and highly sold potted flowers in China. Horticultural enthusiasts around the world have produced varieties with good characteristics. However, there are still problems among varieties of *Oxalis* in China, such as the unknown origin of some resources, confusion in nomenclature and unclear classification. In addition, the lack of testing techniques and standards for new varieties in China has hindered the promotion and application of Chinese *Oxalis* and has seriously discouraged breeders. Therefore, it is necessary to develop DUS test guidelines for new varieties of *Oxalis* in China, to describe the characteristics and features of new varieties of *Oxalis* and to determine their DUS. This is also of great significance in regulating the existing varieties of *Oxalis* and protecting the legitimate rights of breeders.

In this study, the resources of *Oxalis* were collected and preserved for three consecutive years in 2018, 2019 and 2020, 185 representative *Oxalis* were selected as experimental materials, and 6–10 of each variety with good growth status were selected for repeated measurement. Through the methods of introduction and cultivation, data query, field observation and quantitative taxonomy, phenotype observation and diversity analysis were carried out, and the DUS testing technology of new varieties of oxalis was systematically developed, in order to provide scientific basis for the identification, evaluation, breeding and protection of *Oxalis* species.

Materials and methods

Plant materials

The germplasm resources of *Oxalis* were collected and organized through seed introduction and market purchase, and planted in the *Oxalis* germplasm resource at College of Landscape Architecture, Fujian Agriculture and Forestry University, and 185 varieties of *Oxalis* materials were finally identified for the development of the DUS test guidelines. The selected materials had been cultivated and observed for three years (2019.07–2022.03), including 60 native species and 125 varieties (online Supplementary Table S1).

Experiment methods

Information collection

We followed the guidelines by the UPOV guidance documents for the Testing Guidelines Preparation (TGP series of documents) (UPOV, 2002; Organization, 2017), general guidelines for testing for DUS of new plant varieties (State General Administration of quality Supervision, Inspection and Quarantine. General guidelines for testing distinctness, uniformity and stability of new plant varieties, 2004), general introduction for testing for DUS of new plant varieties (Tang, 2017) and Japanese test guidelines for DUS of *Oxalis* (www.hinshu2.maff.go.jp). We consulted the Flora of China and overseas, plant taxonomic studies, DUS test guidelines for related plants in China and abroad and varieties descriptions, as well as websites related to *Oxalis* (Pacific Bulb Society Oxalis, Plants of the World Online, Scottish Rock Garden Club Forum) (Lonsdale and Leven-Latvia, 2007; Govaerts, 2018; Alla and Olga, 2020) combining the morphological characteristics, breeding information, production applications and scientific research of the *Oxalis* in China. The relatively important descriptive characteristics were preliminarily screened (online Supplementary Table S2).

Field observation

The field observations of characteristics were mainly carried out according to the observation period, observation site and observation method. Except for young leaf colour, which was observed at the young leaf period, all other leaf characteristics were observed at the leaf maturity period. Flower characteristics were observed at the flowering period, and plant subterranean characteristics were observed at the plant dormancy period. Numerical quantitative characteristics (QN) such as plant height, plant crown width, leaflet length and leaflet width were observed using vernier callipers or straightedge measurements to find their average values (online Supplementary Figs S1 and S2). Qualitative characteristics (QL) and pseudo-qualitative characteristics (PQ) were measured visually. The colour characteristics such as flower colour and leaf colour were observed using the Royal Horticultural Society colourimetric card. To minimize errors, all colour measurements were done by the same person and the colour card code was recorded.

Data processing

Based on the field observations of the phenotypic characteristics of *Oxalis*, the data of phenotypic characteristics measured in three consecutive years were summarized and counted using Microsoft Excel 2019. Through grading and assigning values of QL and PQ, the number and frequency of distribution of each characteristic in the population were counted. For QN, this study calculated their average value, extreme value, median value, range (R), standard deviation (SD) and coefficient of variation (CV), and classified the QN that met the requirements of DUS into five grades. By referring to the probability grading method (Liu, 1996), as X is the average value and SD is the standard deviation, we defined (X–1.2818SD), (X–0.5246SD), (X + 0.5246SD) and (X + 1.2818SD) as four points, so that the probabilities of occurrence of the five levels were 10, 20, 40, 20 and 10%, respectively. When the distribution range of values was small, two points, (X − 0.5246SD) and (X + 0.5246SD), were further divided into three levels.

Correlation analysis and R-type cluster analysis were performed for each characteristic using Origin 2021 software to screen out suitable characteristics. The correlation analysis of QN was performed using the raw measurement data, while the correlation analysis of QL and PQ was performed after assigning values to the raw data of each characteristic. R-type cluster analysis was performed using the between-groups linkage method, Euclidean distance and *k*-means clustering algorithm, and a tree clustering diagram was drawn, based on the results of correlation coefficients of QN, QL and PQ.

Results

Preliminary screening of the test characteristics

Based on the preliminary data collection and field observations, 130 characteristics were screened for testing, including four plant characteristics, seven above-ground main stem characteristics, 46 leaf characteristics, 13 inflorescence characteristics, 55 flower characteristics and five underground seed bulb characteristics. Among the characteristics collected in the preliminary period and the characteristics tested in the DUS test guidelines of *Oxalis* published in Japan, some characteristics were not easily distinguishable or difficult to observe, although they were important in plant taxonomy. To make the test guidelines more operational, some characteristics that did not apply to DUS test guidelines in China for the *Oxalis* were excluded.

It was found through long field observations that the characteristics of the underground seed bulbs of *Oxalis* were not easy to observe, and the size of seed bulbs was very unstable in different species within a variety (online Supplementary Fig. S3). In addition, the improper operation would lead to damage or death of the whole plant. For example, 'occurrence location of seed bulb', 'with or without traction root' and 'thickness of the underground part of the main stem' were not easy to observe and were not important ornamental parts. The bulb sizes of the same species or varieties vary greatly and are very unstable (online Supplementary Fig. S3). In addition, no spots or markings were found on the petioles of any of the *Oxalis* observed in the field. Therefore, the characteristics including 'petiole: marking', 'petiole: marking colour', 'petiole: marking type' and 'petiole: number of marking colours' were excluded. The characteristic 'flower: closed at night', which was not conducive to distinguishing between species, was excluded, as it was a common phenomenon in *Oxalis* and lacked polymorphism. Some species or varieties of *Oxalis* had striped or scattered spots on the lower surface of petals, but only had one colour for spots (online Supplementary Fig. S3) and, therefore, 'petal: spot stability' and 'petal: number of spot colours' were excluded. As the *Oxalis* are mainly ornamental in pots, the advantages and disadvantages of ornamental characteristics are most important in the characteristics of the flowers and leaves in the above-ground parts of the plant. Therefore, based on the observation of each characteristic, and by the method specified in TG/29/7, this guideline adopts some of the characteristics of Japanese test guidelines for DUS of *Oxalis*. Meanwhile, we excluded the above characteristics that did not apply to the observation of *Oxalis* in China, and added some characteristics, such as plant: crown width, leaves: bearing mode, petiole: whether have or not, etc., to the initial screening of 120 characteristics, aiming to make the test guideline more operable and applicable.

Quantitative characteristic classification and analysis

Variance analysis

The CV reflects the variation of each phenotypic characteristic within and among populations. The smaller the CV (<15%) among individual plants within a population is, the more stable the characteristic, which is beneficial for determining the consistency of new plant varieties. A higher CV among populations reflects the greater degree of dispersion of the characteristic, which not only helps to distinguish different germplasms but also facilitates the development of grading standards for QN and the identification of key breeding targets. The overall CV for the 28 QN ranged from 7.68 to 31.2% within species or varieties and from 20.74 to 97.23% between species or varieties (online Supplementary Table S3). The CVs for 22 characteristics, including 'plant: height', 'plant: crown width' and 'main stem: above-ground thickness', were small within species or varieties, all less than 15%, while the CVs between species or varieties were large, all larger than 25%. This indicates that these characteristics were stable within species or varieties and met the requirements of the DUS test, while the germplasm could be clearly distinguished between species or varieties. Therefore, these 22 QN were provisionally included in the DUS test characteristics of new varieties of *Oxalis*. The CV within species or varieties for 'inflorescence: diameter', 'inflorescence: number of flowers' and 'inflorescence: number' all exceeded 15%. We retained them for the time being, because their CVs between species or varieties were greater than 36%, which was not only representative in the grading of QN, but also had a greater impact on the ornamental value of *Oxalis*. The characteristic of 'main stem: above-ground length' was excluded. Its measuring range included 'main stem: above-ground inter-nodes length' and 'main stem: above-ground inter-node length' and had a large CV within species or varieties (15.11%), which was unstable. The characteristic 'only for single-flowered species: number of single flowers' was also excluded as it showed the highest CV among the *Oxalis*, both within and between species or varieties, which was very unstable and did not meet the requirements for consistency in DUS testing.

Quantitative characteristic classification

Based on the calculation results, the grading range of each characteristic was obtained. The eight characteristics 'main stem: above-ground thickness', 'inflorescence: length', 'inflorescence: diameter' and 'inflorescence: number' were divided into three grades, and 18 characteristics such as 'plant: height', 'plant: crown' and 'compound leaf: width' were divided into five grades (online Supplementary Table S4).

Quantitative characteristic correlation analysis

The results of the correlation analysis of QN showed that although there were some correlations among the QN, most of the characteristics showed light colours in the matrix (online Supplementary Fig. S4) and the absolute correlation coefficients of the characteristics were generally low (<0.5). Only 40 pairs had correlation coefficients above 0.6. Thus, the characteristics screened in this study were reasonable, representative and independent. Because there are many characteristics, we extracted some of the characteristics for analysis. The extracted characteristics were representative ones and those having the high or significant correlation coefficients. 'plant: height' and 'main stem: above-ground length' showed considerably positive correlations. The correlation coefficient of 'plant: height' and 'main stem: above-ground length' was 0.536, which indicated that these two characteristics were closely related. As the plant height increased, the above-ground length of the main stem also increased. The correlation coefficients between 'plant: crown width' and 'compound leaf: length', 'compound leaf: width', 'petiole: length' were significantly positive, reaching 0.477, 0.444 and 0.473, respectively. This indicates that the size of plant crown is usually influenced by the size of compound leaves and the length of petiole. As the compound leaves get larger and the petioles get longer, the occupied plant area and the plant crown width get larger. The correlation coefficients between 'main stem: above-ground thickness', 'main stem: above-ground length', 'main stem: above-ground villi' and 'main stem: above-ground branch' were all in the range of 0.496–0.777 between every two characteristics. Among them, the correlation between the 'main stem above-ground villi' and the 'main stem above-ground branch' was the strongest, with a correlation coefficient of 0.777. It indicated as the occurrence of branches on the above-ground main stem become more, the villi on the main stem become more. However, although these four characteristics were closely correlated with each other, the correlation levels among these four characteristics and other quantitative characteristics were much lower. The correlations among 'main stem: above-ground length', 'petiole: length' and 'petiole: thickness' were considerable and negative, with correlation coefficients of 0.466 and 0.429. This is mainly because some of the *Oxalis* have above-ground main stems, while some do not, and the petioles are either very short or do not have petioles. Those *Oxalis* with long above-ground main stems have either very short petioles or no petiole. Therefore, there is a considerably negative correlation between the above-ground length of main stems and the length and thickness of petioles. There was a considerably negative correlation between 'leaflets: width' and 'leaf: degree of inversion along the midrib'. It indicates that a wider leaflet weakens the ability of the leaf inverse along the midrib, which is consistent with the actual field observations. The correlation coefficients between the characteristics 'inflorescence: length', 'inflorescence: diameter', 'inflorescence: number of flowers', 'inflorescence: number', 'pedicel: length', 'pedicel: thickness' and 'pedicel: hardness' were all ⩾0.8. In addition, 'inflorescence: length' and 'inflorescence: diameter' were closely related to 'inflorescence: thickness', 'inflorescence: number of flowers' and 'inflorescence: length', and the correlation coefficients were as high as 0.922, 0.911, 0.895, 0.834, 0.894 and 0.836, respectively. The decision to exclude or retain can be based on the subsequent R-type clustering.

Quantitative characteristic R-type cluster analysis

Since all characteristics are correlated, it is not possible to judge whether to exclude or retain a characteristic based on the correlation coefficient alone. Therefore, on the premise of analysing the correlation of QN, the correlation coefficient was used to systematically cluster the characteristics, and the correlation analysis and clustering results were combined to consider whether to exclude characteristics with strong correlation.

From the R-type clustering dendrogram (Fig. 1), it can be divided into five major groups, groups A, B, C, D and E when the genetic distance was 1.90. In group A, 'plant: height', 'main stem: above-ground thickness', 'main stem: above-ground villi', 'main stem: above-ground branch' and other seven characteristics were clustered together, indicating that these seven characteristics were considerably correlated. Among them, 'plant: height' and 'main stem: above-ground length' showed considerable correlation in both R-type clustering and correlation analysis. Since 'plant: height' was a combination of several characteristics such as 'main stem: above-ground length', 'pedicel: length' and 'petiole: length', therefore, it was more appropriate to exclude 'main stem: above-ground length', which was more correlated. Considering that the above-ground villi of the main stem were too small to be influenced by personal subjective factors when judging the number of villi and that the villi characteristics were observed on the leaf surface, petiole and flower stalk of the plant, 'main stem: above-ground villi' was excluded to simplify the DUS test.

In group B, although the five characteristics 'leaflets: apex notch depth', 'flower: height relative to leaf', 'plant: number of leaves', 'leaf: vein obviousness' and 'style: height relative to anthers' were clustered into one group, the correlation coefficients of these characteristics were generally low in the correlation analysis, so these five characteristics were retained.

In group C, 11 characteristics were clustered together, such as 'leaflets: thickness', 'calyx: width', 'calyx: thickness', 'petal: thickness ', 'flower stalk: length', 'flower initiation', 'flower stalk: width' and 'flower: diameter'. Among the 11 characteristics, 'calyx: width' and 'calyx: thickness' were close to each other, and they were considerably positively correlated, so just one of them was retained. Since the observed value distribution range of 'calyx: thickness' was too small (0.03–0.25 mm) in the actual field observation and the difference was not considerable, it was considered more appropriate to exclude this characteristic. The difference between 'petal: thickness' and 'petiole: length', 'petiole: width', 'petal: width', 'petal: length' and 'flower: diameter' was strong. Since the value distribution range of 'petal: thickness' was small (0.02–0.14 mm), and there was no considerable difference between species or varieties, it was excluded. Three characteristics, 'petal: length', 'petal: width' and 'flower: diameter' reflected the size of flowers. Among these, 'flower: diameter' was a combination of 'petal length' and 'petal: width', so only the 'flower: diameter' characteristic was retained.

Group D mainly contained seven characteristics: 'plant: crown width', 'petiole: length', 'compound leaf: width', 'compound leaf: length', 'leaflets: width', 'leaflets: length' and 'petiole: thickness', which were closely related to each other and mainly reflected the width of the plant crown and the size of the leaf and petiole. Although 'compound leaf length', 'compound leaf width', leaflets length and leaflet width were related to leaf size, they were all retained because *Oxalis* involves both single and compound leaf types.

Group E mainly contained seven inflorescence-related characteristics such as 'inflorescence: length', 'inflorescence: diameter' and 'inflorescence: number of flowers', and the correlation coefficients of the seven characteristics were as high as 0.684–0.943, indicating that these seven characteristics were highly correlated. However, 'inflorescence: number of flowers' and 'inflorescence: diameter' were excluded, considering that they had large and unstable CV within species or varieties, and had highly considerable correlations with the above inflorescence characteristics. 'pedicel: hardness' was excluded because it was difficult to judge the degree of hardness and softness, and it was influenced by personal subjective factors during field observation.

Quality, pseudo-quality characteristic correlation analysis

The correlation analysis of 39 QL and 33 PQ showed that most of the characteristics had light colours in the matrix heat map (online Supplementary Fig. S5) and the correlation coefficient of each characteristic was low, with only 73 pairs having a correlation coefficient of 0.6 or higher. This indicates that the QL and PQ screened in this study were representative and independent enough to show sufficient differences among varieties. They were also easily distinguishable between known varieties when tested for specificity in new plant varieties. Due to many characteristics, only those characteristics with high correlation coefficients (⩾0.6) and considerable correlations were specifically analysed in this study, while those with small correlation coefficients and only significant levels were not listed.

The correlation among 'above-ground main stem: with or without', 'main stem: above-ground colour', 'leaf: location of occurrence' and 'leaf: the way of attachment' were considerable and positive, with correlation coefficients of 0.865, 0.651 and 0.826. 'Leaf: upper surface villi' and 'leaf: lower surface villi' were considerably correlated, with a correlation coefficient of 0.747. The correlation among 'leaf: upper surface villi', 'leaf: lower surface villi' and 'leaf: closed at night', 'petiole: with or without', 'petiole: villi', 'petal: overlapping', 'petal: spot', 'petal: wavy edge' ranged from 0.594 to 0.706, indicating that with or without villi on leaves was closely related to with or without villi on stalks and petioles in the *Oxalis*. 'Leaf: closed at night' and 'petiole: with or without', 'petiole: villi', 'petal: overlapping', 'petal: spot' and 'petal: wavy edge' were considerably correlated, and the correlation coefficients were 0.731, 0.672, 0.575, 0.646 and 0.657, respectively. The three characteristics 'leaf: spot', 'leaf: spot type' and 'leaf: spot colour' showed considerably positive correlations with each other, with correlation coefficients ranging from 0.874 to 0.940. This indicates that the characteristics of leaf spots were more uniform in the phenotypic expression of *Oxalis*. 'Inflorescence: with or without', 'inflorescence: type', 'peduncle: colour', 'pedicel: colour', 'peduncle: villi' and 'peduncle: villi' all showed highly significant positive correlations with correlation coefficients ranging from 0.746 to 1.000. The highest correlation coefficient (1.000) was found between 'inflorescence: with

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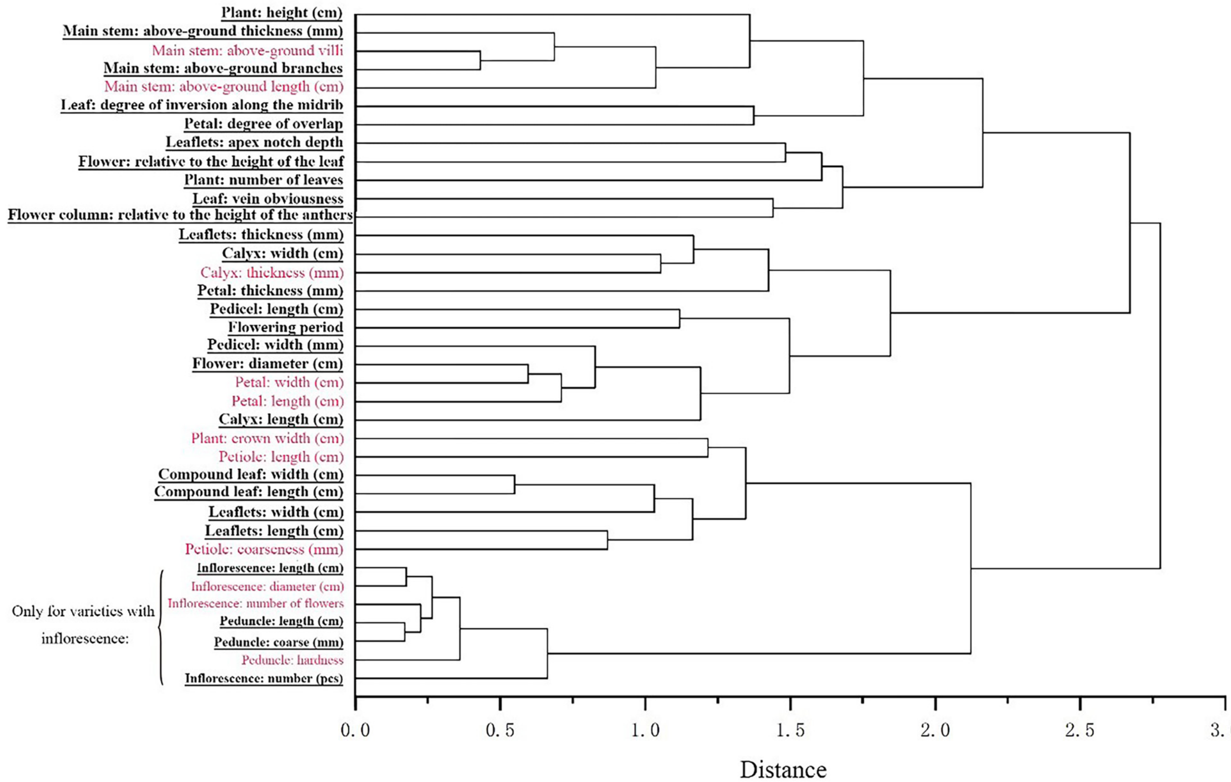

Figure 1. R-type clustering dendrogram of 37 quantitative characteristics. (the non-excluded and finally selected characteristics are distinguished using bold fonts, underlines, while the excluded characteristics are distinguished using red font.).

or without' and 'inflorescence: type', mainly because when the plant had inflorescence, it also had the corresponding inflorescence type. The correlation coefficient between 'peduncle: villi' and 'pedicel: villi' reached 0.968, which is very close to 1.000, indicating their homogeneity in terms of characteristic expression. The correlation coefficient between 'petal spot: type' and 'petal: spot colour' was highly significant and positive, reaching 0.818.

Quality, pseudo-quality characteristics R-type cluster analysis

From the R-type clustering dendrogram (Fig. 2), when the genetic distance was 2.4, 39 QL and 33 PQ could be classified into four categories, namely F, G, H and I, which covered almost all phenotypic characteristics and ornamental points of the *Oxalis*.

The group F mainly included 42 characteristics such as 'seed bulb: shape', 'plant: plant type', 'petal: spot type', 'petal: spot colour' and other 42 characteristics. The correlation coefficients of 'petal: spot type' and 'petal: spot colour' were as high as 0.818, and the two characteristics were observed on the same site, which showed that these two characteristics were highly correlated. Considering that the expression status of 'petal: spot colour' was relatively close, and its colour was usually the same colour family and had weak differentiation; while the characteristic 'petal: spot type' has considerably expression differences among varieties, so 'petal: spot type' was retained and 'petal: spot colour' was excluded. 'Leaf: upper surface colour' and 'young leaf colour' were clustered together and showed a considerably positive correlation, both of which also reflected the overall leaf colour. Since the colour of the upper surface of mature leaves reflected the colour of young leaves in an integrated manner, 'young leaf: colour' was excluded, and 'leaf: upper surface colour' was retained. 'Calyx: shape' and 'calyx: state' were close to each other, and had some correlation. In addition, the shape of the calyx reflected the state of the calyx comprehensively. It means when the calyx is lanceolate in shape with an acute base, it is in an inversion state, and when the calyx is oval, it is usually spreading. Therefore, only 'calyx: shape' was retained. 'Leaf dorsal: heterochromatic' was excluded as this characteristic was clustered with 'leaf dorsal: heterochromatic colour' and both had a high correlation coefficient of 0.913. The observed site was the same, and the expression statuses of 'leaf dorsal: heterochromatic colour' was usually the same colour family.

The group G mainly included 11 characteristics such as 'above-ground main stem: with or without', 'main stem: above-ground colour', 'leaf: the way of attachment' and 'leaf occurrence location'. Among these, although 'above-ground main stem: with or without' and 'main stem: above-ground colour' were clustered together, they were not excluded because the expression status of the two characteristics was very different.

The group H mainly included ten characteristics such as 'leaflets apex: notch with or without', 'leaf: closed at night' and 'leaf: upper surface villi'. Among them, 'leaflets apex: notch with or without' and 'leaf: closed at night' were clustered into a small group with strong correlation, mainly because the leaves closed at night were mostly heart-shaped, and the leaves with sinus presence at the leaflets apex were also mostly heart-shaped. Given the different nature of the two field observations, they were not streamlined.

The group I mainly included nine characteristics such as 'leaf: spot', 'leaf: spot colour' and 'leaf: spot: type'. Among them, although 'only for varieties with inflorescence: peduncle: villi', 'only for varieties with inflorescence: pedicel: villi', 'only for

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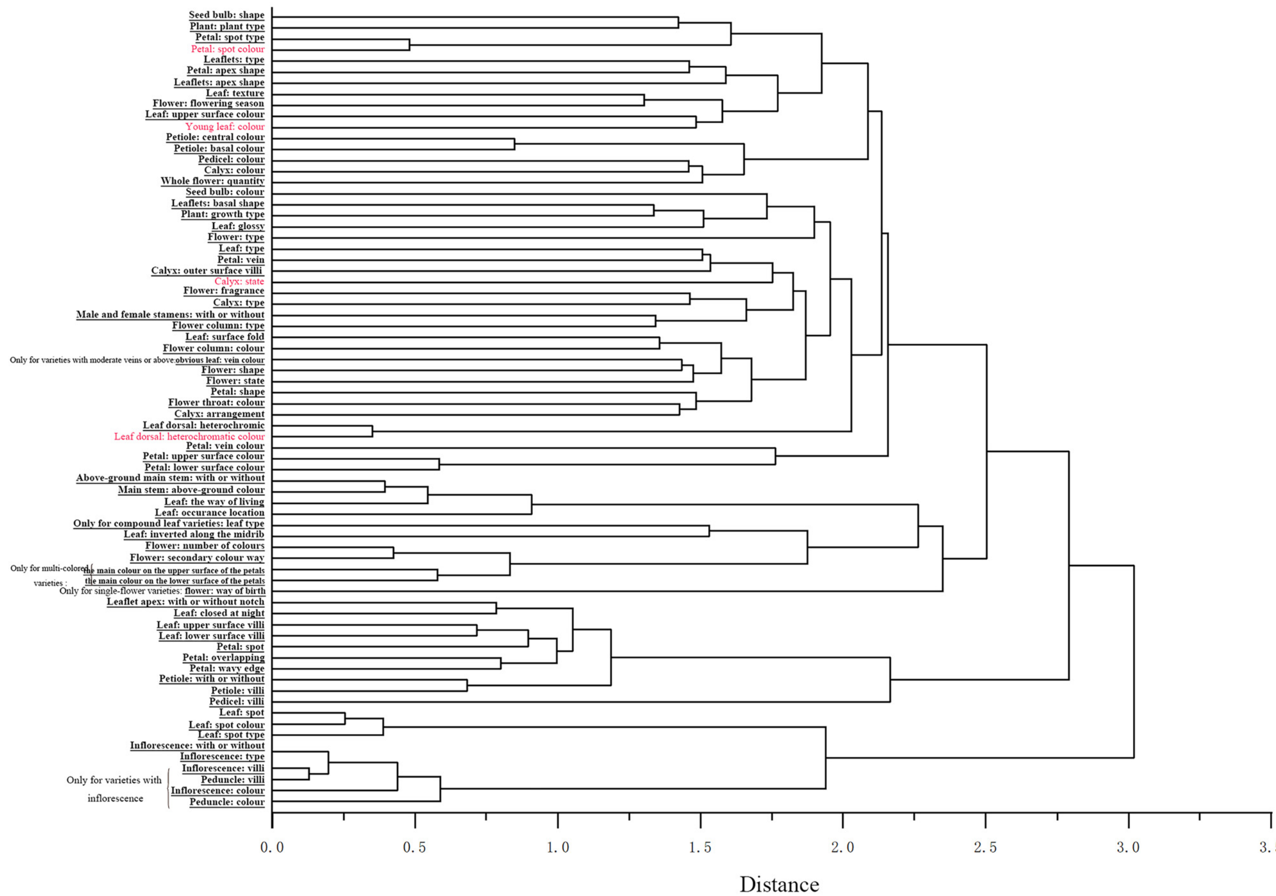

Figure 2. R-type clustering dendrogram of qualitative characteristics and pseudo-qualitative characteristics. (the non-excluded and finally selected characteristics are distinguished using bold fonts, underlines, while the excluded characteristics are distinguished using red font.).

varieties with inflorescence: peduncle: colour', 'only for varieties with inflorescence: pedicel: colour' were highly correlated, but were not appropriate to be excluded, as the observed parts were not the same.

Grouping characteristic screening

The screening of grouped characteristics should select QL or characteristics whose performance was important for determining variety specificity in different locations. At the same time, the selected group trait should be as many as possible. If fewer varieties meet the criteria of a trait, the trait is more appropriate for a group trait of the varieties. Based on these principles and the stability and ease of observation of the characteristics, seven characteristics were selected to be grouped, including 'plant: plant type', 'inflorescence: type', 'flower: flowering season', 'flower: the way of attachment', 'flower: the way of growth', 'flower: the way of attachment', 'petal: upper surface colour', 'flower: fragrance' and 'above-ground main stem: with or without'.

Standard species determination

In the test guideline, to clarify the different expression statuses of a characteristic, the corresponding characteristic expression status generally corresponds to a standard variety. The standard varieties mainly played a reference role in characteristic classification and were used to assist in judging the reliability of the test. In selecting standard varieties, the following principles were followed: (1) stable characteristics, less affected by environmental factors; (2) existing known varieties; (3) widely distributed and easy to obtain and use; (4) accurate and consistent characteristic performance and characteristic description; (5) control on the number of standard varieties, not too many, and screening of known varieties that can represent multiple characteristic expression statuses; and (6) characteristics that can be illustrated by pictures do not need standard varieties. According to these principles, 26 standard varieties were finally determined in this guideline (online Supplementary Table S5).

Discussion

The DUS test guideline for new species of the *Oxalis* in China were based on an extensive review of the relevant literature on the *Oxalis* at home and abroad (Ornduff, 1987; Dong *et al.*, 2020). Based on a survey of the main plant species in the market, and a long-term botanical survey and morphological observations, combining the morphological characteristics, resource evaluation, breeding, production applications and the actual situation of scientific research in China, 96 characteristics with stable expression and easy observation were selected as DUS test characteristics. The 96 characteristics included 28 QN, 38 QL and 30 PQ. Compared with the Japanese test guideline, this guideline added 19 characteristics with potential for DUS testing, including 'plant: crown width', 'leaf: the way of attachment', 'petiole: with or without', 'inflorescence: with or without', and excluded 36 characteristics including 'seed bulb: size', 'cotyledon: occurrence location', 'mother ball: persistence', 'seed bulb: preservation time' and 'seed bulb: firmness'. A high proportion of these characteristics was related to subterranean characteristics and physiological characteristics. They were tedious to observe and not only reduced the efficiency of the test, but were also not the main characteristics to distinguish ornamental *Oxalis*. 'leaf: serrated edge' was not found to be serrated during the development and observation of this guideline. Therefore, the current establishment of this characteristic lacks practicality in some sense. In addition, this guideline also adjusted the names of some characteristics and their expression status. For example, in the Japanese test guideline, the length of the erect stem to the ground surface was usually referred to as the lower part of the main stem, while the part of the erect stem branched at the ground surface was referred to as the above-ground part of the main stem. To make the test guideline more operational and widely applicable, this guideline had unified these two characteristics to the above-ground part of the main stem. As ornamental herbs with great value, the ornamental characteristics of *Oxalis* were most important in terms of plant, flower and leaf characteristics. Therefore, the selection of test characteristics should focus on ornamental characteristics, species status, breeding level and direction.

Although Japan pioneered the DUS test guideline for new species of *Oxalis* and provided more details in testing characteristics, some characteristics did not apply to China. In this study, we combined the market demand and breeding needs of the ornamental *Oxalis* and developed a guideline for testing ornamental *Oxalis*. Our guideline is more comprehensive in testing characteristics and more detailed in expression status, and most of the resources have been covered in the development of this guideline, thereby providing a wide range of applications.

In recent years, correlation analysis and cluster analysis have been widely used by most scholars in the process of characteristic screening for DUS test guidelines. For example, in the development of the DUS test guideline for *Ipomoea aquatica* varieties, 34 test characteristics were finally screened using correlation analysis and other methods, which provided a scientific basis for the DUS test of *I. aquatica* varieties and the substantive review of variety rights (Zhang *et al.*, 2021). The results of the correlation analysis of 12 QN of *Hippeastrum* clearly showed that 'flower: diameter' had a considerably positive correlation with most of the characteristics, which led to the screening of this characteristic as the selected test characteristic (Zhu *et al.*, 2016). Yu (2019) applied correlation analysis and cluster analysis to 16 QN of 75 *Euphorbia pulcherrima* and revealed that the correlations among the characteristics were generally small, which indirectly justified the screened characteristics. In this study, the correlation analysis and cluster analysis were applied to 37 QN, 39 QL and 33 PQ for characteristic screening, and the correlation degree among characteristics was clarified. Moreover, 'above-ground main stem: length' and 'petal: length' and other characteristics that were closely related to the other characteristics were excluded, which not only reduced the workload of measurement but also reduced the error in future DUS tests. Therefore, the results of correlation analysis and cluster analysis can be fully used to screen and validate each characteristic index in the future DUS test guideline characteristic screening.

The lack of unified taxonomic standards, confusing nomenclature and unclear species classification are prevalent issues among the introduced *Oxalis* species in China. These problems significantly hinder the conservation, utilization, classification and identification of *Oxalis* germplasm resources in China, as well as impede hybrid breeding and production promotion efforts for *Oxalis*. In the process of market circulation, the expression status of *Oxalis* plants from different sources usually varies, and the known species are mixed, resulting in homonymy and heterosis. Therefore, it is particularly important to evaluate, differentiate or identify them using some unified method (e.g. DUS test guidelines), which has been used in *Brassica napus* (Lai *et al.*, 2013), *Helianthus annuus* (Shan *et al.*, 2020), and has been proven to be effective in its application. In the present study, morphological observations of many *Oxalis* using the DUS test guideline also revealed some synonyms or synonymous species. For example, *Oxalis nidulans* and *Oxalis fabaefolia* are both called the bean leaf in market. This shows that the DUS test guidelines can be effectively used for resource evaluation and is of great relevance.

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