Received: 22 August 2022 | Accepted: 29 November 2022

DOI: 10.1002/aps3.11513

SOFTWARE NOTE

Applications in Plant Sciences

Multiple Leaf Sample Extraction System (MuLES): A tool to improve automated morphometric leaf studies

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Abstract

Premise: The measurement of leaf morphometric parameters from digital images can be time-consuming or restrictive when using digital image analysis softwares. The Multiple Leaf Sample Extraction System (MuLES) is a new tool that enables high-throughput leaf shape analysis with minimal user input or prerequisites, such as coding knowledge or image modification.

Methods and Results: MuLES uses contrasting pixel color values to distinguish between leaf objects and their background area, eliminating the need for color threshold–based methods or color correction cards typically required in other software methods. The leaf morphometric parameters measured by this software, especially leaf aspect ratio, were able to distinguish between large populations of different accessions for the same species in a high-throughput manner.

Conclusions: MuLES provides a simple method for the rapid measurement of leaf morphometric parameters in large plant populations from digital images and demonstrates the ability of leaf aspect ratio to distinguish between closely related plant types.

KEYWORDS

aspect ratio, breadth, Feret diameter, leaf shape, morphometrics, shape analysis

The quantitative analysis of form, termed morphometrics, is a useful methodology with many applications in biology, including genetics (Zhu et al., 2020), ecology (McKee et al., 2019), evolution (Shi et al., 2019), and agriculture (Huang et al., 2018). In plant biology, morphometric analyses are commonly used to obtain measurements and examine variability in plant organs, especially leaves. One of the traditional uses of leaf shape has been in distinguishing species and subspecies across and within geographic regions. Indeed, plant identification guides used by both researchers and the general public rely heavily on leaf shape variation to distinguish otherwise similar plants (Rushforth and Hollis, 2006; National Audubon Society, 2021). Plants have distinct leaf shapes, which can be used to determine to which species, subspecies, or cultivar a given plant belongs. In addition, variability in functional leaf traits, such as leaf size and shape, has been shown to be linked to differential plant responses to environmental pressures (Pérez-Harguindeguy et al., 2013; Schrader et al., 2021). Leaves are the main photosynthetic organs in plants, and differences in these functional leaf traits are known to have an impact on plant productivity, including effects on total yield (Fan et al., 2015; Cui et al., 2017), seed size (Huang et al., 2018), and aboveground biomass (Digrado et al., 2022), highlighting the importance of morphometric analyses for these particular plant organs.

Traditionally, morphometric analysis has been a laborious process involving hand measurements taken in the field. When studying leaf morphology, the process of collecting leaves and performing measurements (e.g., leaf length, width, area, and shape) can be tedious, particularly in large populations (Schrader et al., 2021). Recently, methods for measuring leaf shape and size have shifted away from hand measurements and toward digital image analysis tools, especially when performing leaf collections ex situ (Chitwood et al., 2014; Migicovsky and Myles, 2017). As computational analysis has become standard in morphometric analysis, numerous tools have been developed to aid in the process; however, many of these require prior coding knowledge or are manual or semi-automatic by design. These softwares require constant user interaction or preprocessing of leaf images or scans, resulting in an error-prone and time-consuming process when working with large data sets or populations. To address some of these issues, we have used previously developed methods as precedent to create a tool for the rapid quantification of traditional morphometric parameters for simple leaf shapes: the Multiple Leaf Sample Extraction System (MuLES). The MuLES tool generates measurements of traditional morphometric parameters, such as leaf length, width, shape, area, and aspect ratio, in a high-throughput, automated manner. Due to its automated implemention, MuLES does not require user coding knowledge and provides a simple graphical user interface (GUI) for users to select their desired parameters, making it easy for any researcher to use. In this paper, we demonstrate the effectiveness of MuLES in extracting high-throughput data from several crop species: blueberry (*Vaccinium* L. sect. *Cyanococcus* A. Gray), cranberry (*Vaccinium macrocarpon* Aiton), citrus (*Citrus* L. spp.), and cowpea (*Vigna unguiculata* (L.) Walp.) (Figure 1A–D). We further demonstrate its ease of use and discuss applications of the extracted data for further analyses, including trait mapping.

METHODS AND RESULTS

Leaf measurement terminology

Of the traditional morphometric parameters in leaf morphology studies (i.e., leaf length, width, size, shape, and aspect ratio), two commonly measured parameters are leaf length and width. Researchers have measured these in different ways, however, especially when using digital image analysis softwares. For example, "length" is defined in Leaf Analyzer (Weight et al., 2008) as being measured vertically from the leaf apex down to the petiole attachment (base) of the leaf using a straight line, whereas for the MASS software (Chuanromanee et al., 2019) it is defined vertically in a straight line from the base and through the centroid of the leaf. "Width" is defined in LeafJ (Maloof et al., 2013) as the longest horizontal line perpendicular to the length line, and alternatively, for the MASS software, as a horizontal line through the centroid of the leaf, perpendicular to the length line. This lack of standard definitions can be a source of confusion in measuring leaf length and width, and may

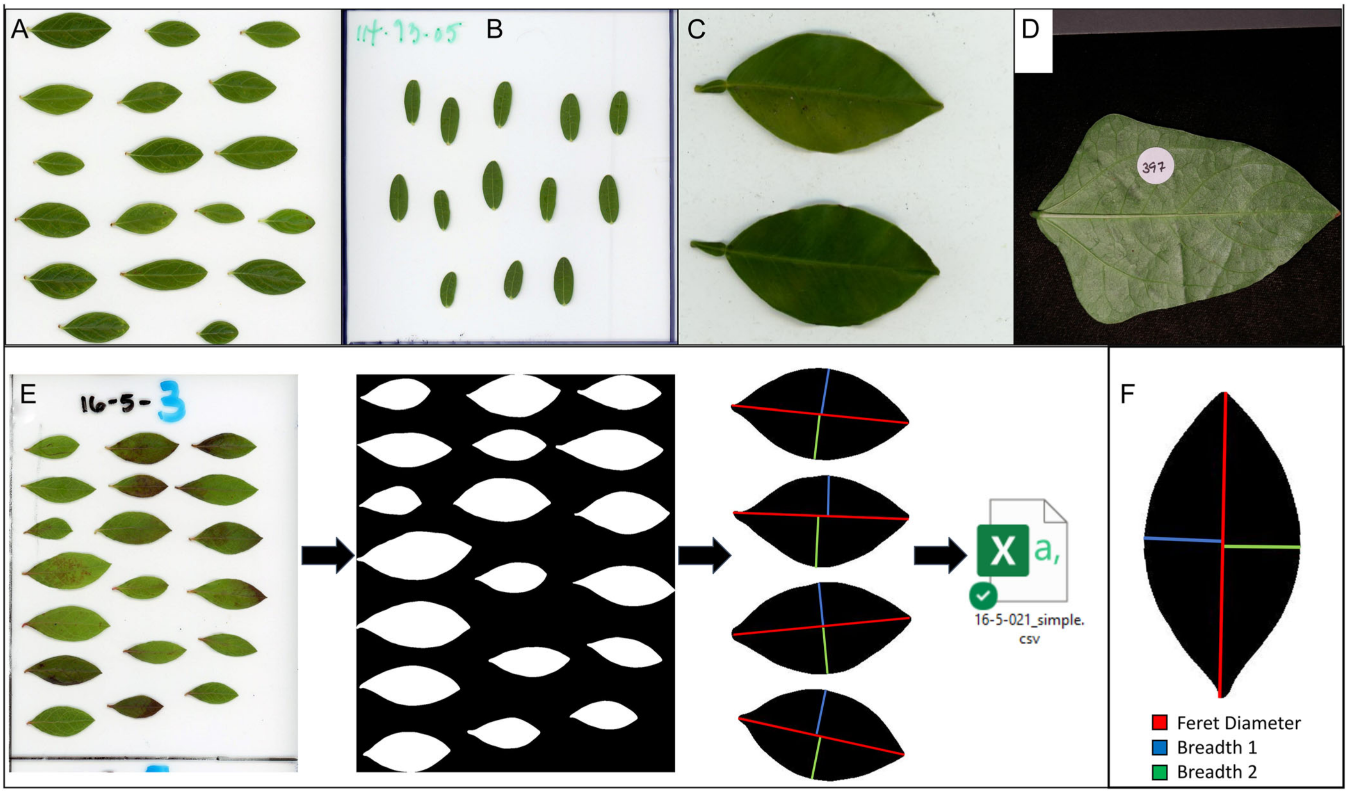


FIGURE 1 Examples of leaf input images: (A) blueberry, (B) cranberry, (C) citrus, and (D) cowpea. (E) Demonstration of MuLES workflow using blueberry leaves: Raw scans containing multiple leaves are processed (left), producing cutouts of each leaf image (center), which are measured for a variety of parameters and output to a .csv file based on user-specification (right). (F) Parameters measured by MuLES include the Feret diameter (red line) and breadth (blue and green lines).

introduce unexpected differences in measurement values depending on the software used. Furthermore, this may distort analyses due to the inherent asymmetricality of leaves caused by environmental effects (e.g., drought stress, disease, pests).

We suggest the use of more definitive terminology when discussing leaf measurements using digital image analysis. Here, we propose using the Feret diameter and the breadth when performing digital measurements. Figure 1F is a visual example of how the Feret diameter (red line) and the breadth (green and blue lines) are measured using MuLES. The Feret diameter, also known as the maximum caliper or caliper diameter, is defined as the longest distance between any two points along the boundary of an object and is typically applied in microscopy when measuring particle sizes (Merkus, 2009). Additionally, we adopt the definition of breadth as described by Landini (2008) as the largest axis perpendicular to the Feret diameter that may be an additive measurement of two lines (Figure 1F, green and blue lines) (Landini, 2008). This entails measuring the breadth as the sum of two lines from the two widest points on the boundary perpendicular to the Feret diameter, rather than as a single perpendicular line through the Feret diameter. By measuring in the proposed manner, MuLES provides more accurate measurements of leaf width in most simple leaf types than those based on a single line measurement spanning the width and/or breadth of the leaf (which may be an underestimation or overestimation). This method of measuring both the right and left sides of leaves accounts for variability in leaf shapes due to environmental (e.g., pests or diseases) or genetic factors (e.g., oblique lobes), and has been previously described (Freeman et al., 1994).

Software

The MuLES tool was written as a simple macro script to measure the basic morphometric parameters of leaves in a two-dimensional space. The tool was developed using the open-source image analysis software Fiji (Schindelin et al., 2012). Fiji is a pre-packaged distribution of ImageJ (Schneider et al., 2012) that includes several plugins geared toward scientific image analysis and is available on Windows, Mac, and Linux operating systems. The MuLES tool is available under the GNU General Public license v3.0 and can be found at https://github.com/0cb/mules. Currently, MuLES is compatible with Fiji 2.3.0/1.53f51 and Java 1.8.0_172 on Windows (10 and 11) and Linux (Ubuntu 22.04), and Fiji 2.3.0/1.53q and Java 1.8.0_202 on macOS (Monterey 12.0.1), but is expected to function properly with more recent versions of Fiji and Java than those listed. This tool is dependent on the Morphology and Biovoxxel plugins available in the ImageJ library (Landini, 2008; Brocher, 2022).

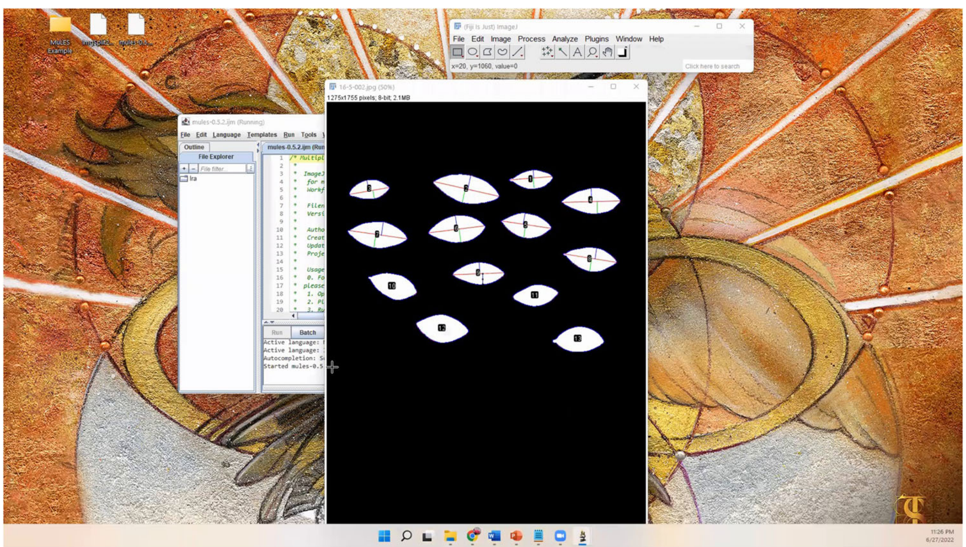
The MuLES tool includes a GUI that facilitates ease of use among users without strong coding backgrounds (Appendix S1). The workflow requires minimal user interaction (e.g., manual thresholding or defining leaf objects) and is fully automated to analyze multiple images containing multiple leaves. This software does not apply a color thresholding step typically found in other shape analysis softwares, e.g., Easy Leaf Area (Easlon and Bloom, 2014), LeafJ (Maloof et al., 2013), LAMINA (Bylesjö et al., 2008), LeafAnalyser (Weight et al., 2008), and MASS (Chuanromanee et al., 2019). The tool only requires that the background be a contrasting color (preferably white) from the leaves during the imaging process. This allows for the use of leaves that may exhibit discoloration for any reason, such as maturity or environmental influences. This is visible in Figure 1E, where all of the blueberry leaves are recognized by MuLES despite the presence of leaves containing red pigmentation. The fully automated nature of MuLES enables rapid processing of large data sets common in genetic mapping populations.

The MuLES tool begins with user selection of the folder containing the leaf images and the desired folder that output files should be written to. Images used as inputs should be taken under consistent conditions at a sufficiently high image resolution to ensure accurate analysis, taking care to remove potential background noise, such as shadows from inconsistent lighting or folds in the leaf that may detract from certain features, as these will affect the precision of the tool. For this demonstration, we used a flatbed image scanner (Epson Perfection V600 Photo; Epson, Tokyo, Japan) set to 300 or 600 dots per inch (dpi) to ensure consistency across the blueberry, cranberry, and citrus populations. This system is capable of processing images containing a single leaf or multiple leaves and does not require leaves to be positioned in a specific orientation. Users will need to specify certain parameters in the GUI before running the tool, including the input file format (which is case-sensitive), how the results should be output (masked images, quantitative measurements, or both), and the desired image output file format (.jpg or .png). A scale for calibration may also be specified at this point by measuring the distance between two points on a reference feature (e.g., ruler) within an image or via the manual input of known values. This function enables users to designate a single image for determining the appropriate scale for calibration, rather than requiring users to specify the reference feature and scale for each image (assuming image capture conditions are consistent throughout the workflow). Additional parameter options are available and are further described in the "Introduction to MuLES" documentation (available on GitHub, see the Data Availability Statement) and Appendix S2.

After the initial interaction, MuLES will process and analyze each image of the specified format within the specified input folder. Images are first binarized to create a black-and-white image representing the exterior and interior of the leaf, respectively (Figure 1E), before applying a watershed algorithm for irregular shapes to separate individual leaves that may lie in close proximity (Brocher, 2022). Regions (leaves) of interest are determined based on the black-and-white image, followed by analysis of basic morphometric parameters (Landini, 2008). Based on the set parameters, the output can be visualized and saved for individual leaves when selecting “Masks only” or “Both” for the “Desired outputs?” option. These visualizations can be tweaked to include colored representations of the Feret diameter (red) and the breadth (blue and green) of each leaf, as specified by the “Colored Feret diameter and breadth lines?” option. The output(s) produced from the MuLES tool are binarized images of individual leaves and/or measurement data in a .csv file. The binarized leaf images can be used to curate or filter any erroneous data and can be used as inputs for other image analysis tools, such as Momocs (Bonhomme et al., 2014). Given the automated nature of the tool, extraneous objects may be incorrectly recognized as “leaf” objects and may distort analyses; however, a standard deviation–based filter has been implemented to combat this. When selected, this filter is applied to all detected objects within the image and will identify objects to be recommended for removal based on the standard deviation of their measured aspect ratios, making it a useful tool to eliminate background noise and non-leaf objects, as well as removing leaves that may pose as apparent outliers to statistical analysis. The output measurement data include the computed Feret diameter, breadth, area, and aspect ratio (Feret diameter/(breadth 1 + breadth 2)) of the leaves in a .csv format for easy importation into softwares like Microsoft Excel or R. For additional information and examples, please refer to the “Introduction to MuLES” documentation included in the GitHub repository. We have also provided walkthroughs of MuLES (Video 1) as well as a macro script called imgSplit that splits images containing multiple samples into single-sample images for use in MuLES (Video S1, Appendix S3).

Plant materials

Leaves from multiple crop species and accessions were collected to investigate variation within and among means of individuals of each species, as well as to test the application of MuLES in crops with simple leaf shapes. Tested species include hybrid blueberry (an interspecific mapping population developed from a cross between *Vaccinium corymbosum* L. [large leaf] and *V. darrowii* Camp [small leaf]), cranberry (*V. macrocarpon*), cowpea (*Vigna unguiculata*), and several citrus cultivar groups (*Citrus* spp.). Cranberry leaves were divided into two subpopulations, “normal” and “pumpkin” (in reference to the fruit shape), which were visually associated with a distinct obtuse or ovate leaf shape, respectively. Citrus leaves were divided into three subpopulations by cultivar group: citron, mandarin, and pummelo. Example leaf images are shown in Figure 1A–D. Leaves were collected from mature blueberry (summer 2021) and cranberry (autumn 2021) plants maintained in greenhouses located at the Philip E. Marucci Blueberry and Cranberry Center for Research and Extension in Chatsworth, New Jersey, USA (39.72°N, 74.51°W). Leaves were collected from mature cowpea (summer 2019) and citrus (autumn 2021) plants grown in the field at the University of California, Riverside (Riverside, California, USA; 33.97°N, 117.33°W). In this study, only the central leaflet from each cowpea leaf was used due to the observed inherent asymmetricality between the lobes of the lateral leaflets. The number of leaves sampled for each accession varied by species, except in cranberry where the number per accession was consistent. In general, the goal was to sample as many mature leaves as possible for each species, with a maximum of 30 leaves per plant. Leaves from blueberry, cranberry, and citrus accessions were scanned using an Epson Perfection V600 Photo Scanner at 300 or 600 dpi and a CameraTrax 24 Color Card (5.08 × 7.62 cm; https://www.cameratrax.com/) for scale and color correction. Central leaflets for cowpea were pressed before photographing to ensure flatness, then photographed using a Canon EOS Rebel T6i (300 dpi; Canon, Tokyo, Japan) at a 90° angle under consistent lighting conditions with the leaflets positioned on a black velvet background to minimize glare.



VIDEO 1 Walkthrough of the MuLES macro script demonstrating the use of MuLES on scanned blueberry leaf images.

Statistical analysis

To analyze the data from MuLES, the output results table for each accession was analyzed using R to calculate the mean aspect ratio and standard deviation within each accession. As leaf shape is a complex trait that is highly affected by environmental effects, stringent filtering is necessary. If, after removal of obvious outliers, the minimum number of leaves per accession was not met, then the entire accession was discarded. After filtering, an average of 16 leaves were used for each of the 75 blueberry accessions, ranging from 10 to 28 leaves; 12 leaves were used for each of the 62 cranberry accessions, except for one accession where 11 leaves were used; an average of 20 leaves were used for each of the 71 citrus accessions, ranging from 10 to 25 leaves; and an average of 15 central leaflets were sampled for each of the cowpea accessions, ranging between 10 to 24 leaves.

In the cranberry and citrus populations, two-sample *t*-tests were performed comparing subgroups to determine if significant differences were present between those subgroups. Comparisons of subgroups within species showed distinctive patterns of aspect ratio (Table 1). In cranberry, the subgroups were based on two distinct types of leaf shape (normal and pumpkin); the "pumpkin" leaf shape, associated with plants bearing a distinctive pumpkin-shaped fruit, in reference to the fruit's distinctive creases, was shorter and squatter than the "normal" leaf shape. The mean aspect ratios of the two subgroups were significantly different from one another, as determined by a two-sample *t*-test ($t = 9.85$, $P = 5.4e\text{-}14$). The "normal" leaf subsection of the cranberry population revealed an outlier falling within the "pumpkin" range. In citrus, the three tested subgroups (citron, mandarin, and pummelo) also showed significant pairwise differences: citron and mandarin ($t = 7.45$, $P = 1.5e\text{-}12$), citron and pummelo ($t = -6.13$, $P = 6.0e\text{-}09$), and mandarin and pummelo ($t = -20.97$, $P = 2.2e\text{-}16$). As shown by the statistically significant variation observed between cultivar groups in citrus and phenotypic groups in cranberry (Figure 2), aspect ratio can be used to reliably distinguish between closely related plant types. This offers a new tool for group identification when confronted with an unidentified plant. Although only leaf aspect ratio has been evaluated in this paper, the other traits measured by MuLES (leaf length, width, area, shape, roundness, circularity [or roundness index; RI], and solidity) have been demonstrated in the literature to be effective in characterizing leaf shape for genetic, environmental, and evolutionary studies (Chitwood et al., 2014; Shi et al., 2019; Gupta et al., 2020).

TABLE 1 Plant materials tested using MuLES.

Species	Subsection	No. of accessions tested	Total no. of leaf samples	LAR Mean	LAR SD
Blueberry	NA	75	1187	2.50	0.33
Cranberry	Normal	48	575	2.52	0.19
Cranberry	Pumpkin	14	168	1.93	0.13
Citrus	Citron	11	183	1.97	0.28
Citrus	Mandarin	14	324	2.21	0.29
Citrus	Pummelo	46	1073	1.88	0.30
Cowpea	NA	89	1695	1.51	0.05

Note: LAR = leaf aspect ratio; NA = not applicable; SD = standard deviation.

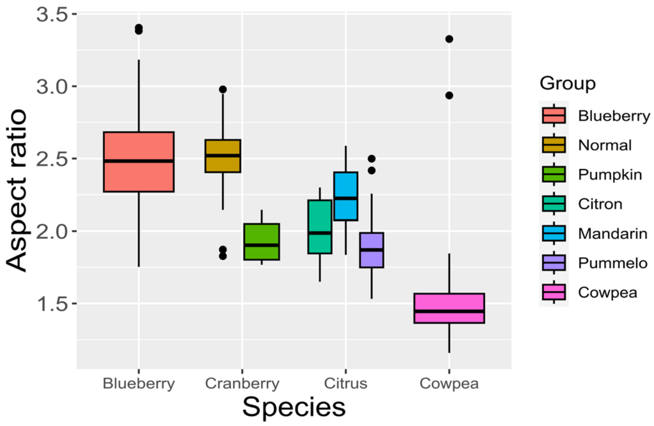


FIGURE 2 Box plot showcasing the leaf aspect ratio distribution among the leaf samples for each plant species. The leaf aspect ratio is defined as length/width. "Normal" and "pumpkin" refer to different types of cranberry plants based on fruit shape. "Citron," "mandarin," and "pummelo" refer to different species within the citrus family.

CONCLUSIONS

The MuLES program simplifies and automates the process of measuring basic morphometric parameters, enabling measurements to be taken for larger plant populations with a few clicks in a timely and efficient high-throughput manner. This tool has successfully identified leaves on the various backgrounds tested using digital images of leaves collected ex situ. In all cases, non-leaf objects were successfully identified and removed, including color cards, labels, and other objects captured in the images. Moreover, MuLES is a versatile tool in the sense that it can be used with a wide range of inputs and data sets, such as digital scans of herbarium samples, that are not compatible with other software prerequisites (e.g., color correction cards, non-green leaf samples). A comparison of MuLES against a few other leaf morphometric softwares, such as MASS (Chuanromanee et al., 2019), MorphoLeaf (Biot et al., 2016), and LAMINA (Bylesjö et al., 2008), is shown in Appendices S4 and S5. As MuLES was written for use in the open-source software ImageJ (and its distributions), the program is free, easily accessible to most users, and does not require background knowledge in coding. MuLES is a simple tool to use and allows researchers to calculate traditional leaf morphometric parameters in an efficient, rapid manner.

These parameters can be used to better understand the role that plant genetics, environment, and their interaction (G × E) play in determining leaf shape. Additionally, the output data and images from MuLES can be exported to other tools for measuring or defining more advanced morphometrics, such as leaf serration (sinuses and contours), elliptical Fourier descriptors, or landmarks. In this manner, MuLES may operate as an intermediate step for those looking to perform outline (e.g., elliptical Fourier analysis) or landmark-based (e.g., Procrustes analysis) geometric morphometric analysis, and can be used for analyses like species discrimination based on leaf shape descriptors or evolutionary models. Data imported into Momocs in this manner have proved to be highly efficient, with no noted loss or misinterpretation of defined objects (Appendix S6). These quantitative data can be used in a variety of studies, complementing studies that require large data sets, including potential associations between leaf morphometric parameters and certain genetic factors. Future genetic mapping analyses will allow for elucidation on relationships between leaf shape parameters generated by MuLES and other traits in many plant species.

At present, MuLES has only been validated using the simple leaf shapes of the species in the present study. Preliminary work has been done to test the efficacy of MuLES with lobed leaves using a publicly available leaf data set (Silva et al., 2013) and has been found useful in correctly measuring pinnately lobed leaf types, but unreliable in measuring palmately lobed leaf types. MuLES measures the farthest points in a straight line to determine the Feret diameter, and therefore is unable to distinguish the apex of the leaf from the tip of a lobe. This limitation may extend to leaves that diverge from a typical ellipse shape. Leaves that exhibit certain base types such as sagittate, hastate, or auriculate, where the base of the leaf may extend past the petiole attachment of the leaf, as well as leaves where the midvein exhibits a high degree of curvature are subject to incorrect measurements (Appendix S7). Future work will be required to validate and expand the usage of MuLES in other plant species with more complex leaf shapes or unique features. A drawback that may be noted for MuLES is that errorless automation can be difficult to achieve, and although a filtering feature has been included to combat this, the feature itself has its limitations and may also remove "true" leaf objects that happen to have an aspect ratio that lies outside of the standard deviation of objects in the image. When processing images containing a single leaf, the standard deviation filter is able to remove background noise, but is not capable of expanding its range to leaves in other images and therefore is unable to remove those outlier leaves from measurements, thus requiring filtering in the post-analysis. As with any image analysis tool, it is recommended that users test the MuLES tool on a subset of their images to ensure that the selected features are producing the expected output (e.g., user-defined standard deviation limit, output image colors) before running MuLES with the entire data set to minimize runtimes and troubleshooting.

AUTHOR CONTRIBUTIONS

The project was conceptualized by C.S.B, Z.J., N.V., and I.A.H. The program was written by C.S.B. Data collection and analysis were performed by R.T., X.W., S.P.K., S.L, and I.A.H. C.S.B wrote the manuscript with input from all other authors. All authors approved the final version of the manuscript.

ACKNOWLEDGMENTS

The authors thank Timothy J. Close (University of California, Riverside) for providing the imaging station used for the cowpea and citrus leaves. The project was partially supported by the U.S. Department of Agriculture National Institute of Food and Agriculture (USDA NIFA FACT grant 2019-67022-29930 to Z.J.), as well as the New Jersey State Land Grant Allocation and the Specialty Crop Research Initiative (SCRI-NIFA Award 2019-51181-30015, VacciniumCAP to N.V.). S.L. was supported by the University of California President's and Chancellor's Postdoctoral Fellowship Program.

DATA AVAILABILITY STATEMENT

The MuLES and imgSplit scripts are open source and freely available on GitHub (https://github.com/0cb/mules), along with a detailed introduction ("Introduction to MuLES") and sample leaf images from each species tested. Video demonstrations of the MuLES macro script (Video 1) and imgSplit macro script (Video S1) are included with the article and are also available on YouTube (MuLES: https://www.youtube.com/watch?v=vtj93rbDO28; imgSplit: https://www.youtube.com/watch?v=9HVsNvAWPjE).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Appendix S1. The MuLES graphical interface.

Appendix S2. Description of the MuLES scale calibration and additional parameter options.

Appendix S3. Demonstration of the imgSplit function. (A) Input image from blueberry with four accessions scanned simultaneously. (B) The four output images after splitting. Each image will be saved individually and can be used with the MuLES tool.

Appendix S4. Preliminary comparison of measurement methods between MuLES and other leaf morphometric applications.

Appendix S5. Comparison of MuLES with other leaf morphometric applications.

Appendix S6. Comparison of image data when importing MuLES outputs into Momocs.

Appendix S7. Examples of various leaf shapes and their compatibility with MuLES: (A) *Alnus* sp., (B) *Populus alba*, (C) *Betula pubescens*, (D) *Urtica dioica*, (E) *Quercus robur*, (F) *Tilia tomentosa*. Leaf parameters are subject to error when the lobes or base extend farther than the petiole attachment (B, F). Image contents are as follows (left to right): full color image of input leaf, outline as determined by MuLES, morphometric measurements as determined by MuLES.

Video S1. Walkthrough of the imgSplit macro script. The video demonstrates the usage of imgSplit on a combined scan of multiple blueberry leaf samples to produce individual images.

How to cite this article: Bowman, C. S., R. Traband, X. Wang, S. P. Knowles, S. Lo, Z. Jia, N. Vorsa, and I. A. Herniter. 2023. Multiple Leaf Sample Extraction System (MuLES): A tool to improve automated morphometric leaf studies. *Applications in Plant Sciences* 11(2): e11513. https://doi.org/10.1002/aps3.11513