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[1]Moscow South-West High School (N 1543), 119571, Moscow, 26 Bakinskikh komissarov str., 3–5. Russian Federation
[2]Moscow State University, Department of Biology, Vorobyevy Gory, Moscow, Russian Federation, 119899
[3]Minot State University

POLINA A. VOLKOVA[1]; SOF'YA A. KASATSKAYA[2]; ANNA A. BOIKO[2] & ALEXEY B. SHIPUNOV[3]

# Stability of leaf form and size during specimen preparation of herbarium specimens

With 4 Figures

**Keywords:** geometric morphometry, herbarium specimens, morphology, taxonomy

Abstract

It is common to draw taxonomic conclusions from the analysis of herbarium material and then apply them to living, fresh material. However, some widely used morphological characters are changed during the preparation of specimens and therefore the applicability of morphometric data obtained from the measurement of dried plants is not universal. We tried to estimate quantatively how different leaf characters change during drying. Fresh and dried leaves of ten plant species were measured and scanned to determine how the stability of commonly used absolute (e.g., leaf length) characters, relative characters (e.g., relative position of maximal width) and leaf contour. For the latter, we employed landmark-based thin plate spline (TPS) analysis. We found that all absolute sizes differed significantly after drying (typically decrease original size 0.9–0.8 times), whereas leaf shape did not differ significantly between fresh and dried leaves of most species. Relative characters were more stable then absolute ones but less stable than a form as a whole. We argue that it is essential to provide measures, contours and/or photographs of fresh leaves together with herbarium material, all the more so because in two cases (20%) leaf contour also significantly changed after drying.

## Introduction

Most research on plant taxonomy is still made on the basis of herbarium material. The advantages of herbarium specimens over fresh plants for the systematist are evident; study of herbarium material widens the geographical area of the investigation, minimizing time and financial costs. Herbarium specimens constitute a taxonomic resource for botanical specialists that may be used after collection for dozens or even hundreds of years. The existence of herbarium material allows botanists to consult with the specialists for a long time after collection (BRIDSON & FORMAN 2004). However, some important diagnostic characters, e.g., size and shape of the leaves, can change during the drying process and in theory could decrease the diagnostic power of these characters, when living and dried plants are compared. Unfortunately, despite the potential validity of problem, investigations of leaf deformation resulting from the preparation of herbarium specimens are rare. Changes of leaf size have been investigated, for example, in several European species of dactylorchids (*Dactylorhiza* Necker ex NEVSKI, see VERMEULEN 1947). The geometric morphometric approach provides a set of contemporary tools allowing calculation of shape as a whole (ROHLF 2006, 2007) and therefore should be useful in studies of specimen preparation processes. The only such investigation known to the present authors is that by VOLKOVA (2008) who employed these methods in a study of three closely related

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*Nymphaea* species (*Nymphaea alba* L., *N. candida* Presl and *N. tetragona* Georgi) and showed that the leaf shape of a species can change substantially after drying, and may even become more similar to the shape of the dried leaf of another species. This phenomenon could be more widespread and occur not only in aquatic plants. Therefore a more detailed investigation of leaf deformation during specimen preparation is desirable, employing more plant species.

Morphometric characters differ in their ability to reflect plant form. Some mostly provide information about size, whereas others also contain information related to shape (BOOKSTEIN 1989; ZELDITCH et al. 2004). Moreover, landmark- and curve-based characters may contain almost "size-free" information (ADAMS et al. 2004), i.e. two leaves with the same shape would be treated as identical, although their linear sizes could differ substantially. These character types may react differently to the drying process. We investigated the behavior of these character types in order to clarify their relative stability, and also to provide researchers with information which could be useful in the preparation of herbarium collections for subsequent investigations.

## Materials and methods

Since only herbaceous plants with relatively thick leaves have so far been used in studies of specimen preparation (VERMEULEN 1947; VOLKOVA 2008), we decided to add more species with different leaf characteristics, including herbaceous and woody plants. We added two aquatic species: *Potamogeton perfoliatus* L. (Potamogetonaceae) and *Polygonum* (*Bistorta*) *amphibium* L. var. *aquaticum* Hook. (Polygonaceae); and eight terrestrial species, herbaceous and woody: *Plantago media* L., *P. lanceolata* L. and *P. major* L. (Plantaginaceae), *Polygonum (Bistorta*) *bistorta* L. (Polygonaceae) *Ribes nigrum* L. *and R. spicatum* E. Robson (Saxifragaceae / Grossulariaceae), and *Alnus incana* (L.) Moench and *A. glutinosa* (L.) Gaertn. (Betulaceae). These plant species are widespread in Europe, easy to obtain and therefore to make it possible to reproduce our results more easily. Their leaf structures (simple, undivided or lobed) are compatible with the chosen method of geometric morphometrics, thin-plate spline (TPS); this analytical approach does not work well with compound, extensively dissected leaves or perforated leaves (SHIPUNOV & BATEMAN 2005). Our ten species are members of five genera (*Potamogeton, Plantago, Polygonum* (or *Bistorta*), *Ribes* and *Alnus*) where the size and shape of leaves are important distinguishing characters. In several groups, there are taxa, species or subspecies (with sometimes questionable taxonomic identity) where the distinction is largely based on leaf characters, e.g. *Alnus kolaensis* Orlova (often regarded as an ecological form) differs from *A. incana* by smaller ovate-ovoid leaves with an obtuse tip (NILSSON 2000; ILINSKY & SHIPUNOV 2005); *A. barbata* C.A. MEY differs from *A. glutinosa* by more elongate leaves (ILINSKY & SHIPUNOV 2005); *Potamogeton praelongus* Wulfen can be distinguished from *P. perfoliatus* by larger and more elongated leaves (DANDY 1980; PAPCHENKOV & SCHERBAKOV 2003); *Plantago media* subsp. *stepposa* (Kuprian.) Sóo differs from *P. media* subsp. *media* by more elongated lanceolate leaves (GRIGORJEV 1958; SHIPUNOV 2000).

Plants were sampled in the Tver region of Russia (Udomlya district) in the end of June 2006 and 2008. We sampled leaves from 20 to 80 plants per species; in all, 340 plants were sampled. The largest non-damaged leaf was taken from a randomly chosen shoot of each plant. We measured maximum length, maximum width (distance between points of maximum lateral margin curvature) and location of maximum width (Fig. 1B) of the fresh leaf, manually outlined its contour with a pencil, scanned the leaf with digital scanner (300 dpi) and proceeded to prepare specimens. The preparation of herbarium specimens was performed under the usual conditions (BRIDSON & FORMAN 2004) where the layers of drying paper between the herbarium sheets were changed three times a day. The leaves were outlined, scanned and measured again after specimen preparation was complete. The herbarium specimens are deposited in Moscow South-West High School (Russia) and scans of the leaves can be downloaded at: http://herba.msu. ru/shipunov/moldino/leaves.

Thin plate spline analysis, or TPS (ROHLF 2006, 2007; ZELDITCH et al. 2004) was employed for the analysis of leaf shape deformation. The shape of the leaf of *Alnus incana* and *A. glutinosa* was described with 12 landmarks located on the ends of main veins, and also at the tip and the base of the leaf – this method has already been proven as optimal for alder leaves (ILINSKY & SHIPUNOV 2005). For *Potamogeton perfoliatus*, we used 100 equally distant pseudo-landmarks where the first one was located on the leaf tip. The leaf shape of the other 7 species was described with 4–6 landmarks each, specifically placed at the points of the maximal contour curvature (Fig. 1). Coordinates of the landmarks were written to the data file with tpsDig (ROHLF 2006). Consensus configuration, values of principal relative and partial warps (which characterize the degree of differences between the specimen and consensus configuration) were calculated with tpsRelw (ROHLF

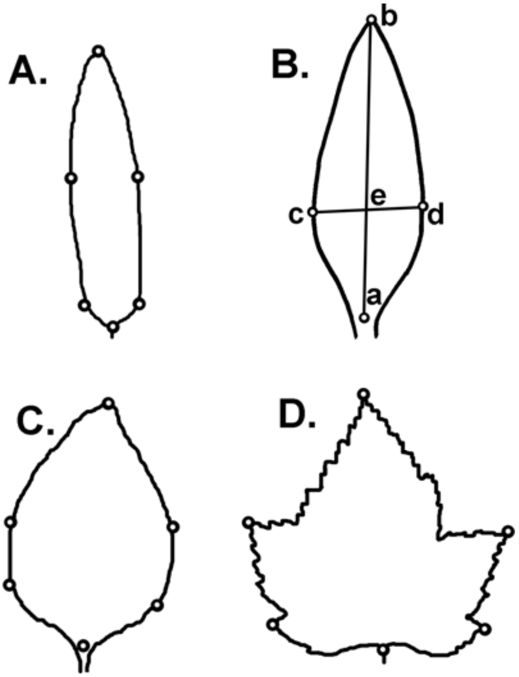


Fig. 1
Typical positions of landmarks used for geometric morphometric analysis (landmarks set at the points of maximum curvature of the leaf contour): A — *Polygonum amphibium* and *Polygonum bistorta*, B — *Plantago lanceolata* and *Plantago media*, C — *Plantago major*, D — *Ribes spicatum* and *Ribes nigrum*. Scheme of the measurements is shown in Fig. 1B: ab — maximum length, cd — maximum width, ae — location of maximum width

2007), which implements TPS analysis in a way similar to principal component analysis (PCA). Original coordinates were normalized by the Procrustes fit method ($\alpha = 0$: ROHLF & SLICE 1990).

Principal component analysis of the relative warps matrix was employed for the classification of leaf shapes, similarly to the way described elsewhere (SHIPUNOV & BATEMAN 2005; ILINSKY & SHIPUNOV 2005). Non-parametric MANOVA ("adonis" analysis: OKSANEN et al. 2007) based on 1000 permutations was used to determine the differences between pre- and post-drying clusters in PCA. All calculations and graphs were made in the R environment for statistical computing (R Development Core Team 2005).

## Results

In all studied leaves, linear sizes decreased significantly during specimen preparation. In the majority of cases, shrinking of leaves varied from 10% to 20% (Table 1, Figs. 2 and 4). In contrast, the relative location of maximum width (location of maximum width to maximum length ratio) changed significantly only for *Plantago media*, and even in this case the difference was small (the ratio was equal to $0.41 \pm 0.05$ before and $0.45 \pm 0.07$ after specimen preparation). The relative length (maximum length to maximum width ratio) changed significantly for all the three *Plantago* species (Fig. 4) and also for aquatic *Polygonum amphibium* and *Potamogeton perfoliatus*. This ratio increased after specimen preparation, i.e. the leaves of these five species became more elongated after drying (Fig. 2). The leaf shape did not change significantly after specimen preparation (Fig. 3) for any species except *Plantago media* and *Potamogeton perfoliatus* (Table 1). In addition, we found that hand-made contours are appropriate for TPS analysis almost to the same extent as scanned images as we did not find significant differences between the results of these two approaches.

## Discussion

The decrease in linear sizes of leaves of different plant species in our study (10–20%) is comparable with the results of VOLKOVA (2008), who found that linear sizes of *Nymphaea* species (*N. alba*, *N. candida* and *N. tetragona*) decreased on average by 30%. The coefficient used by VERMEULEN (1947) for comparison of dry and fresh *Dactylorhiza* plants varied around 15%. Using this range (10–30%) to compare the sizes of fresh and dried leaves of plant species with different morphology and ecology we found that the amount of shrinking does not vary much, being less in woody species and more in water plants (Table 1).

The investigated leaf characters differ in their stability after specimen preparation. Strictly linear measurements (maximum width and maximum length) are the least stable characters. They changed significantly in all studied species. Relative measurements (relative length and relative location of maximum width) which are usually considered to be more shape-dependent, are more stable. Landmark-based interpretation of leaf shape was the most stable

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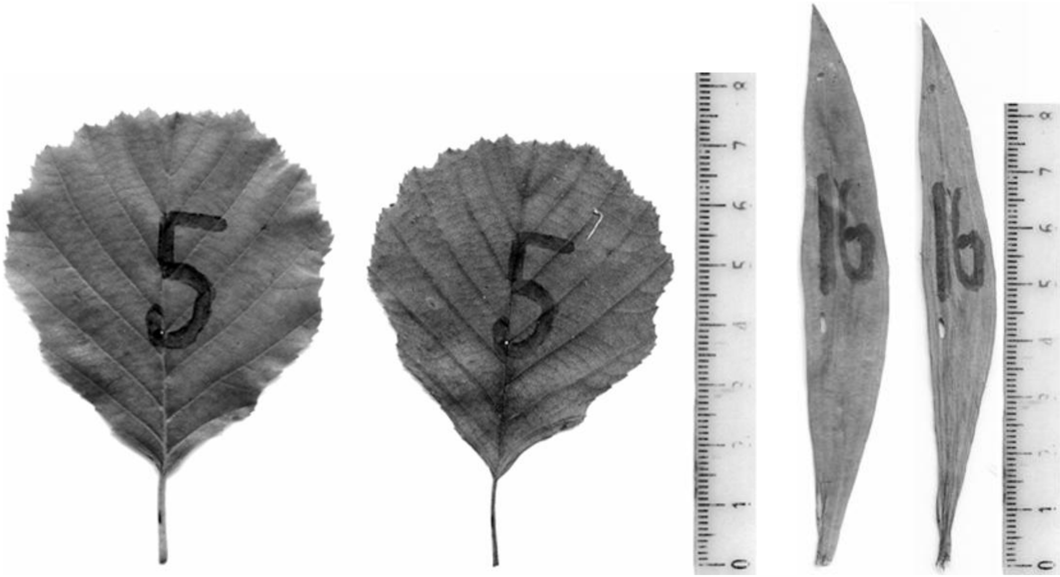
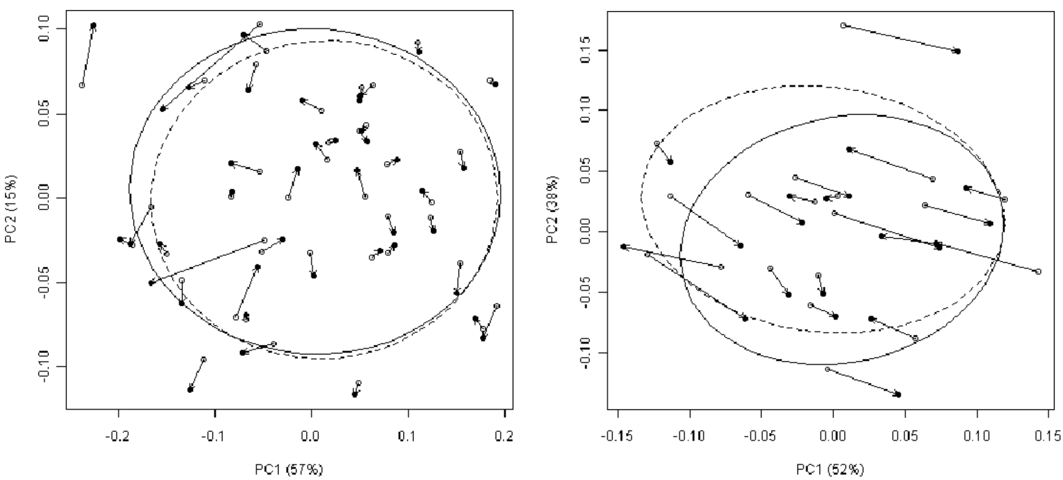


A) B)

Fig. 2
Scanned image of the same leaf before (left) and after (right) specimen preparation (1 cm scale). A — *Alnus glutinosa*: size decreased, shape constant; B — *Plantago lanceolata*: size decreased, shape changed (leaf became more elongated)




A) B)

Fig. 3
Ordination of the analyzed leaves in space of the first two principal components for relative warps of leaf contours: leaf form does not change significantly. Plots show individual leaves ordinated according to their shape within two axes; each leaf was sampled twice, in fresh (open circles) and dried (filled circles) conditions; circles representing same specimen are connected by an arrow. Dashed and solid lines indicate 75% confidence ellipses of leaves' populations before and after specimen preparation, respectively. A — *Alnus glutinosa*; B — *Plantago lanceolata*

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Table 1
Changes of leaf size and shape of the investigated plant species

| Species | Size before (upper line) and after (lower line) herbarization (quartile range, mm) | | Ratio of size after herbarization/size before herbarization[a] | | Significance of differences between leaves before and after herbarization (*p*-values) | | |
|---|---|---|---|---|---|---|---|
| | length | width | length | width | location of max. width/max. length ratio (Student paired test) | max. length/max. width ratio (Student paired test) | relative warps matrix (ADONIS) |
| *Alnus glutinosa* | 53–66<br>51–63 | 49–64<br>46–58 | **0.95 ± 0.03** | **0.94 ± 0.03** | 0.259 | 0.109 | 0.859 |
| *Alnus incana* | 61–73<br>56–69 | 42–50<br>39–46 | **0.93 ± 0.02** | **0.93 ± 0.02** | 0.461 | 0.081 | 0.733 |
| *Plantago lanceolata* | 133–189<br>125–178 | 15–26<br>13–19 | **0.91 ± 0.06** | **0.82 ± 0.06** | 0.302 | **0.001** | 0.851 |
| *Plantago major* | 82–113<br>77–103 | 55–77<br>50–69 | **0.92 ± 0.04** | **0.87 ± 0.04** | 0.292 | **0.001** | 0.884 |
| *Plantago media* | 58–122<br>56–115 | 27–48<br>25–39 | **0.95 ± 0.04** | **0.83 ± 0.05** | **0.045** | **0.001** | **0.014** |
| *Polygonum amphibium* | 100–120<br>91–108 | 26–31<br>24–28 | **0.92 ± 0.02** | **0.89 ± 0.04** | 0.728 | **0.003** | 0.962 |
| *Polygonum bistorta* | 96–124<br>88–114 | 31–42<br>29–38 | **0.93 ± 0.02** | **0.92 ± 0.04** | 0.527 | 0.953 | 0.742 |
| *Potamogeton perfoliatus* | 44–65<br>39–59 | 24–29<br>20–25 | **0.88 ± 0.08** | **0.85 ± 0.07** | 0.432 | **0.003** | **0.001** |
| *Ribes nigrum* | 62–79<br>58–75 | 68–84<br>62–80 | **0.94 ± 0.01** | **0.94 ± 0.02** | 0.810 | 0.328 | 0.940 |
| *Ribes spicatum* | 45–61<br>42–56 | 63–81<br>57–73 | **0.92 ± 0.01** | **0.92 ± 0.04** | 0.164 | 0.992 | 0.884 |

[a] mean ± standard deviation; all the differences before and after herbarization in length and width are significant (Student paired test, $p < 0.05$).

character, since it changed significantly only in two species out of ten. In general, specimen preparation of leaves appears to be a semi-allometric process in which form changed to a much less degree than size.

Leaf shape of the three *Plantago* taxa and the two aquatic plant species (*Potamogeton perfoliatus* and *Polygonum amphibium*) changed similarly after specimen preparation. In all these plants, the leaves became more elongated. This effect was also found in aquatic *Nymphaea* species (VOLKOVA 2008). As previously mentioned, *Potamogeton praelongus* Wulf. differs from *P. perfoliatus* by larger and more elongated leaves; therefore, the difference between herbarium specimens of the first species and newly collected leaves of second species will decrease following specimen preparation. A similar pattern is found in *Nymphaea* where dried leaves of one species became more similar to dried leaves of another species following specimen preparation (VOLKOVA 2008). The factors causing these trends are still unknown. Patterns of leaf deformation after specimen preparation may depend on the position and quantity of mechanical tissues in the leaf. These tissues are known to be less developed in the leaves of aquatic plants (ZALENSKI 1902; POPLAVSKAYA 1948) and in underwater leaves of semi-aquatic plants (GOLIBER & FELDMAN, 1990). Compared with the other investigated terrestrial species with reticulate venation, leaves of the studied *Plantago* species have relatively few arching veins. However, the den-

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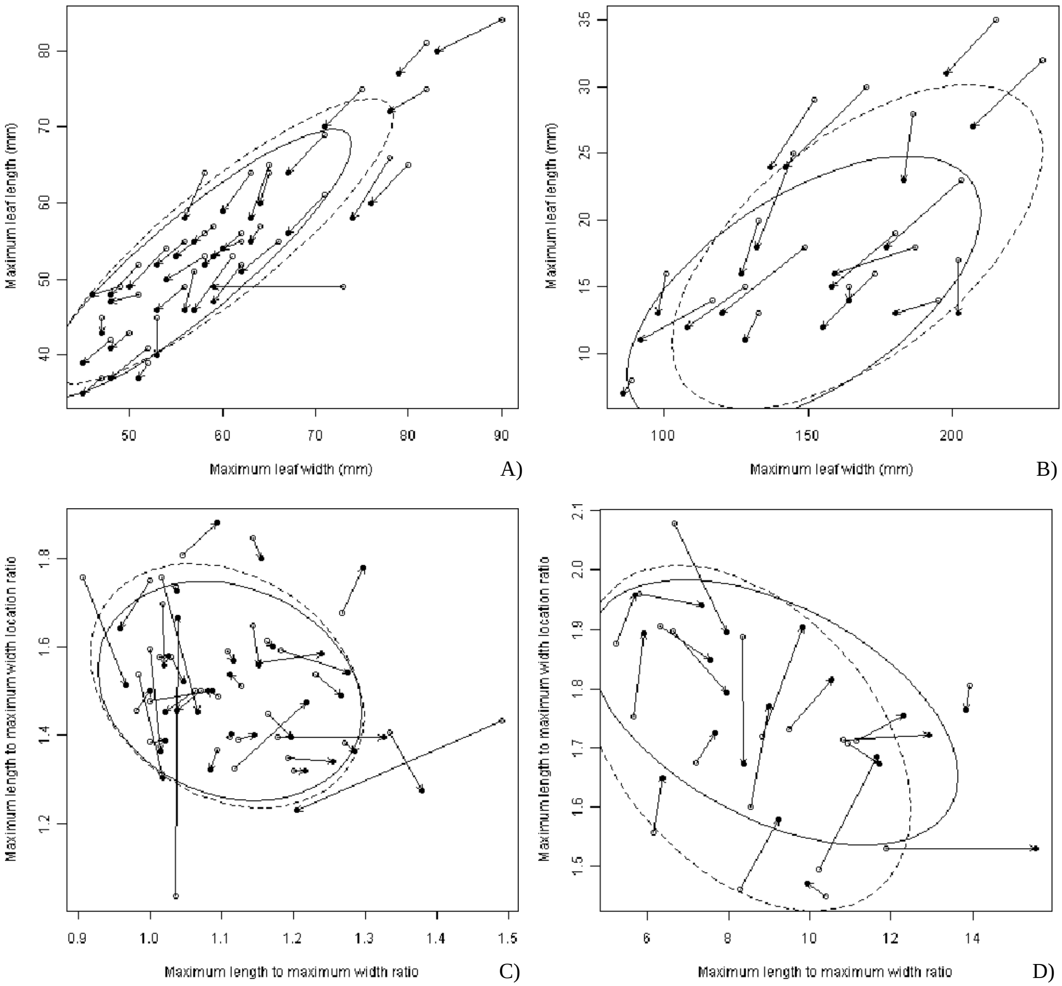


Fig. 4
Change in leaf size after specimen preparation. Plots show individual leaves ordinated according to their shape within the two axes; each leaf was sampled twice, in fresh (open circles) and dried (filled circles) conditions; circles representing same specimen are connected by an arrow. Dashed and solid lines indicate 75% confidence ellipses of leaves' populations before and after drying, respectively. A–B — Absolute characters (maximum width and maximum length): A — *Alnus glutinosa*; B — *Plantago lanceolata*. C–D — Relative characters (relative length and relative location of maximum width): C — *Alnus glutinosa*; (D) *Plantago lanceolata*

sity of veins of the first to third orders in our species with directed leaf deformation was not significantly lower (VOLKOVA & SHIPUNOV, unpublished data). Orientation of veins or density of veins order higher could also be an important factor in these deformations.

In all, our data shows that specimen preparation causes significant linear and (in some cases) shape-related deformation of leaves. This could result in a significant bias between observations made from herbarium specimens and those made in the field and even hamper the discrimination of close species. To avoid this, we recommend the use of more stable (relative and shape-based) metric characters, or the employment of linear characters which have a high (more than 30%) degree of difference between taxa. This is especially important if the taxonomic study is collection-based but results in the creation of diagnostic keys for

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field use. Our results also support the recent practice of supplying photos and measurements of living plants alongwith the herbarium specimen. Outlines of fresh leaves could also help in species discrimination after specimen preparation. These contour outlines require only pencil and paper, and therefore one or two leaf contours might be a simple and useful addition to preparation of specimens from populations in variable environmental conditions.

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Address of corresponding author:

Dr. Alexey B. Shipunov, Minot State University, 500 University Ave W, Minot, North Dakota 58707, USA.
E-mail: dactylorhiza@gmail.com
Phone: (701) 858 3116
Fax: (701) 858 3163

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