An Easy Method of Identifying Herbarium Specimens of *Najas minor* and *N. oguraensis* (Hydrocharitaceae)

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*Najas minor* and *N. oguraensis* (Hydrocharitaceae) are annual submerged plants and are difficult to distinguish owing to their similarities. While the number of anther locules and the size of the leaf epidermal cells differ between the two species, the anther locules are often difficult to observe on herbarium specimens and the effectiveness of using the size of the leaf epidermal cells as a taxonomic key character is currently unknown. We examined the size of the leaf epidermal cells in living plants and in dried specimens to evaluate the effectiveness of this feature in species identification. We first identified the two species by observing the anther locule number and then compared the size of the leaf cells in fixed and dried specimens. To identify plant fragments, we examined differences in epidermal cell length and width depending on the position of the cells within the plant. The length and width significantly differed in both fixed and dried leaves between the species. In particular, the epidermal cells of *N. oguraensis* (> 160 μm) were about twice as long as those of *N. minor* regardless of leaf position. We therefore concluded that cell size, especially length, is a valid method for identifying the species in both fresh and dried condition. This method is effective where precise identifications are necessary, such as in floristic surveys and environmental assessments.

Keywords: Diagnostic characteristics, epidermal cell, morphological measurement, number of anther locule, taxonomy

Two obligate annual aquatic species, *Najas minor* All. and *N. oguraensis* Miki (Hydrocharitaceae), are often confused, despite the urgent need for correct identification of endangered species for conservation and for recognizing invasive species. Both species occur from temperate to tropical areas; *N. minor* is distributed widely in Europe and Asia, and has been introduced to North America, while *N. oguraensis* (described from Japanese specimens by Miki 1935) is limited to Asia (Na & Choi 2012, Kadono 2014, Les et al. 2015). In North America, *N. minor* is invasive and has had a considerable impact on freshwater ecosystems (Meriläinen 1960, Robert et al. 2005, Les et al. 2015, Robert 2019). In Japan, *N. minor* is listed as an endangered species in the Japanese national Red Data Book (Ministry of the Environment 2015) and *N. oguraensis* has been included in many prefectural Red Lists (e.g. Environment Department of Nagano Prefecture, Nagano Environ mental Conservation Research Institute 2014). Therefore, their precise identification is required to conserve them as endangered species or to take measures to control them as invasive species.

The two species have often been misidentified owing to their similar morphologies, and observing their unique characteristics is very difficult when using dried herbarium specimens. One of the most useful characteristics for distinguishing them is the number of anther locules of staminate flowers, which are unilocular in *Najas minor* and quadrilocular in *N. oguraensis* (Miki 1935, Kadono 2014; Fig. 1c, d). The length of the anther...
Fig. 1. Morphological differences between *Najas minor* (a, c, e, g, i) and *N. oguraensis* (b, d, f, h, j); distribution of sampled locations (k). (a, b) Staminate flowers; (c, d) cross sections of staminate flowers and anther locules; (e, f) dried herbarium specimens; (g, h) leaf cells in living plants; (i, j) cells of dried leaves under stereomicroscope; (k) black and white circles on map indicate sampling locations for *N. minor* and *N. oguraensis*, respectively.
locule facilitates determination of the locule number, but the small size of the locules (ca. 1 mm long; Fig. 1a, b), makes size difficult to determine, particularly in herbarium specimens (Kadono 1994, 2014). Leaf size and number and size of serrations, which are cited by some authors as characteristic differences between the species (e.g., Ohwi 1965), are practically overlapping and cannot be used to discriminate between species (Kadono 2014; Fig. 1e, f). Although seed coat structure is generally effective in distinguishing the species of *Najas*, *N. minor* and *N. oguraensis* have the same transversely elongated ladder-like seed surface pattern. Without staminate flowers, the species have been impossible to identify morphologically.

In addition to the number of anther locules, the size of the leaf epidermal cells has been used to distinguish these two species (Miki 1935, Kadono 1994; Fig. 1g, h). In the original description of *Najas oguraensis*, Miki (1935) noted that the leaf epidermal cells of *N. oguraensis* are more than twice as large as those of *N. minor*, although it is unclear if the cells of dried or living plants were measured. If the size of the cells of dried specimens differs between the two species, identification would be facilitated, making it unnecessary to damage the specimens to observe the anthers, and also making it possible to identify plants without staminate flowers. Reevaluating herbarium specimens may also provide new information on distribution. Correlation between the size of leaf epidermal cells and the number of anther locules has not been reexamined since the work of Miki (1935). Furthermore, correlation between leaf cell size and leaf size in the two species remains unclear (Kadono 1994), and whether such differences are clearly observable in herbarium specimens in which epidermal cells shrink during drying. Using cell size to identify species has been questioned (Kadono 1994) or disregarded (Na & Choi 2012, Les et al. 2015).

In the present study, we examined the size of leaf epidermal cell in dried specimens to determine if there were differences between *Najas minor* and *N. oguraensis*. First, we confirmed that differences in the size of the leaf epidermal cells in living plants (cells of *N. oguraensis* are larger than those of *N. minor*) were well correlated with differences in the number of anther locules (quadrilocular in *N. oguraensis* and unilocular in *N. minor*). Second, we compared the size of the same epidermal cells in living and dried plants. We then used our findings to identify fragments of dried plants, such as herbarium specimens. We also examined differences in the size of the leaf epidermal cells in relation to leaf size and position on the plant body.

**Materials and Methods**

*Najas minor* or *N. oguraensis* were sampled from 18 lakes and ponds in Japan, including a pond near the type locality of *N. oguraensis*, from 2017 to 2018 (Fig. 1k and Appendix 1). We collected 6 to 10 plants with staminate flowers from each population, cut each plant at the fifth node from the tip of its shoot, and fixed the fragments in ethanol–formalin–acetic acid (FAA). Subsequently, to determine differences in the cells between different leaf positions within a plant, we also collected two plants from each population to make herbarium specimens. We used absorbent blotting paper that included calcium chloride (manufactured specifically to make herbarium specimens by Nihon Vogue Corp., Tokyo, Japan). The plants were placed between blotting papers, which were then placed between two corrugated boards and a metal corrugated plate for ventilation. The plants were held between the blotting papers and boards using wooden boards and bands and dried for 3–4 days using a self-made dryer with a fan. The samples were identified based on the number of anther locules; unilocular = *N. minor*, quadrilocular = *N. oguraensis*. We identified the samples as *N. oguraensis* when the mature staminate flowers were exerted from the leaf sheath. Slits that separate the quadrilocular anthers are observable before pollen release (Miki 1935). Conversely, we identified *N. minor* while the mature staminate flowers were hidden within the leaf sheath without slits, indicating unilocular anthers. To determine the number of
Fig. 2. Box plots of length of epidermal cells between (a) fixed leaf specimens (FLS), (b) dried leaf specimens (DLS), and (c, d) leaves of dried herbarium specimens (DHS). Significant differences between *Najas minor* and *N. oguraensis* were observed in all leaves, which were measured using Student’s *t*-tests (*P* < 0.001). Letters (a and b) indicate significant differences revealed using Tukey’s test (*P* < 0.001).
Fig. 3. Box plots of width of epidermal cells between (a) fixed leaf specimens (FLS), (b) dried leaf specimens (DLS), and (c, d) leaves of dried herbarium specimens (DHS). Differences between *Najas minor* and *N. oguraensis* were measured using Student’s *t*-tests (*P* < 0.01). Letters (a, b) indicate differences revealed using Tukey’s test (*P* < 0.05).
anther locules more precisely, we prepared paraffin-embedded sections (Moll 1888) of staminate flowers (Fig. 1a–d). Voucher specimens were deposited in the herbarium of Niigata University (Appendix 1).

To determine differences between *Najas minor* and *N. oguraensis*, we measured the leaf epidermal cells of material fixed in FAA and dried specimens from the same plant. We measured leaves from 102 plants in 11 populations (*N. minor*, M-AO, M-MM, M-FN, M-YF, and M-YK; *N. oguraensis*, O-MS, O-NA, O-OF, O-HO, O-SY, and O-HH; Appendix 1). We selected the longest epidermal cells adjacent to the leaf veins in the middle of a leaf (between the base and the tip) and measured the selected cell using a microscope. Subsequently, we made dried and fixed specimens using the methods described above to measure the same cells after drying. The same cell was identified by scratching the adjacent cell with tweezers. The length and width of the target cell was then re-measured. We calculated the percent cell shrinkage for *N. minor* and *N. oguraensis* after drying based on the length and width in fixed and dried material. We measured the second longest leaves from each plant, since the longest leaves had been removed for further morphological analysis (S. Midorikawa, in prep.).

To determine whether the leaf epidermal cell size differed between different leaf positions within a single plant, we also measured the length and width of cells from 15 different dried leaves. We selected leaves whose epidermal cells could be clearly observed using a stereoscopic microscope. Subsequently, five leaves from each of three positions on a plant, near the apex, middle, and base of the main shoot, were collected and measured (see Fig. 2d). We measured the length and width of cells from 540 leaves selected from 36 plants. Because the results from comparisons of cell length between the two species were nearly similar between fixed and dried material, we collected and measured sampled from seven more populations to compare as many dried leaves as possible (*Najas minor*, M-BW, M-SK, M-ET, and M-KE; *N. oguraensis*, O-SZ, O-ST, and O-HA; Appendix 1). We measured the length and width of the longest cell located in the middle between the leaf base and apex in the same manner as for the fixed material. We also correlated cell length and width with leaf length and width, excluding the teeth, for 540 leaves. To include both large and small leaves, we randomly selected leaves from the plants we collected.

In all datasets, we determined the equality of variances using the O’Brien test, the Brown-Forsythe test, Levene’s test, and Bartlett’s test ($P < 0.05$). We then used the Student’s $t$-tests to determine significant differences between the two species for both fixed and dried material. For the
dried samples, differences in cell length and width between the leaves near the apex, middle, and base of the shoots were evaluated using Tukey’s test after two-way analysis of variance (ANOVA). In the analysis of cell size versus leaf position, we excluded two populations, O-SZ and O-ST, from which only fragmental samples were collected because the leaf position was unclear (Appendix 1). The software program JMP v.11.2.0 (SAS Institute Inc., Cary, North Carolina, U.S.) was used for the analysis.

Results

We re-measured the leaf epidermal cells of 33 dried specimens prepared from 50 fixed specimens of *Najas minor* and 28 dried specimens from 52 fixed specimens of *N. oguraensis*. We did not measure the remaining fixed material because we could not locate the same cells in the dried material because of deformation of the leaves due to drying. We did measure the same dried and fixed cells in at least three individuals per population and obtained datasets from all populations for statistical analyses.

The longest fixed epidermal cells in *Najas minor* were 85.9 ± 12 (64–120) µm long and 41 ± 8 (24–64) µm wide; those in *N. oguraensis* were 190 ± 28 (144–272) µm long and 58 ± 9 (40–88) µm wide (Figs. 2a & 3a). The longest dried epidermal cells in *N. minor* were 71 ± 12 (48–104) µm long and 26 ± 7 (16–40) µm wide; those in *N. oguraensis* were 173 ± 28 (136–264) µm long and 34 ± 8 (24–56) µm wide (Figs. 2b & 3b). Therefore, both the fixed and dried epidermal cells of *N. oguraensis* were about twice as long as in *N. minor* (Figs. 1g–j & 2a, b; *P* < 0.001). The epidermal cell of *N. oguraensis* were also significantly wider than those of *N. minor* (Fig. 3a, b; *P* < 0.001). The percent cell shrinkage in *N. minor* and *N. oguraensis* was 80.7 ± 8.7% and 91.1 ± 7.1%, respectively.

The longest epidermal cells in leaves near the shoot apex of the dried herbarium specimens of *Najas minor* were 74 ± 14 (40–112) µm long and 28 ± 8 (16–48) µm wide; those of *N. oguraensis* were 165 ± 23 (128–224) µm long and 35 ± 9 (24–64) µm wide (Figs. 2d & 3d). Epidermal cells of leaves near the middle of the shoot of *N. minor* were 72 ± 12 (48–112) µm long and 27 ± 8 (16–48) µm wide; those of *N. oguraensis* were 165 ± 23 (120–248) µm long and 36 ± 14 (16–80) µm wide. Epidermal cells in leaves near the base of *N. minor* were 74 ± 13 (40–112) µm long and 28 ± 8 (5–66) µm wide; those of *N. oguraensis* were 165 ± 29 (120–296) µm long and 37 ± 10 (16–80) µm wide.

Disregarding the position of the leaves, the longest epidermal cells in dried specimens of *Najas minor* were 73 ± 13 (40–112) µm long and 28 ± 8 (5–66) µm wide; those of *N. oguraensis* were 163 ± 24 (120–296) µm long and 35 ± 12 (16–80) µm wide (Figs. 2c & 3c). Significant differences in both cell length and width were observed between these two species (two-way ANOVA, *P* < 0.01), but no significant differences were observed based on leaf position (*P* = 0.90 long and *P* = 0.71 wide). Additionally, no interaction between species and leaf position was observed in either cell length (*P* = 0.87) or width (*P* = 0.53). Tukey’s test showed significant differences in length and width between species (Figs. 2d & 3d, *P* < 0.001).

The leaves of *Najas minor* were 16.88 ± 5.17 (6.48–34.98) mm long and 0.28 ± 0.10 (0.13–0.70) mm wide; those of *N. oguraensis* were 23.12 ± 6.15 (7.16–43.23) mm long and 0.27 ± 0.11 (0.10–0.66) mm wide. Both the length and width ranges of the leaves overlapped between the two species. No correlation was detected between leaf length and epidermal cell length in either species in the analyses based on average sized individuals (*N. minor*, r = 0.13, *P* = 0.62; *N. oguraensis*, r = 0.05, *P* = 0.83) and in all leaf sizes except in *N. oguraensis* (*N. minor*, r < 0.001, *P* = 0.99; *N. oguraensis*, r = 0.133, *P* = 0.03) (Fig. 4).

Discussion

We determined that differences in the size of epidermal cells of the leaves correlated well with
the number of anther locules as Miki (1935) had noted. We determined that dried specimens of *Najas minor* and *N. oguraensis* are distinguishable based on differences in the length of the longest epidermal leaf cells (Figs. 1, 2 & 4). We also noted significant difference in the width of such cells (Fig. 3). Since some fixed leaves shrunk or stretched during drying, we were unable to measure the epidermal cell sizes in some dried material. However, in both cases we were able to divide the specimens into two groups consistent with the two species based on cell morphology.

Since variation in cell width overlapped between the two species, the length of the epidermal cells—which was clearly different between them—should be used for identification. Although the range in length of the epidermal cells did not overlap between the two groups, the maximum value of *N. minor* was close to the minimum value of *N. oguraensis*. Therefore, leaves with epidermal cells > 160 µm long (Fig. 1h) should be sought to identify living, fixed, and dried plants of *N. oguraensis*, since no such cells were observed in *N. minor*. In our study, we measured the cells using a compound microscope, but a stereoscopic microscope can also be used (Fig. 1i, j).

All leaves regardless of their position within the plant can be used for identification, since cell length and width were not significantly different according to leaf position. In field surveys, it is often impossible to collect the whole plant, but by using cell length, fragmented plants of *Najas minor* and *N. oguraensis* can be distinguished. We often observed cell shrinkage in dried plants, probably due to insufficient pressure during the drying process. Therefore, selecting well-pressed leaves is important for easy identification. In addition, the longest cells in some populations of *N. minor* and *N. oguraensis* can be distinguished. We measured only the longest epidermal cells and found that the longest cells in *N. minor* often overlap with the shortest cells of *N. oguraensis* (Fig. 1g, h).

It was previously thought that discrimination between *Najas oguraensis* and *N. minor* using dried specimens was difficult (Kadono 2014). Our findings indicate that leaf epidermal cell size is an effective and easy identification method to identify dried specimens of the two species. Using this method, it is not necessary to damage specimens to observe the number of anther locules, which was previously thought to be the only effective diagnostic characteristic. Therefore, certifying the identity of herbarium specimens is practical using this method, even on specimens without staminate flowers. Precise distribution of these species may be revealed by re-examining herbarium specimens in several herbaria. This method is also effective for activities where precise identification is necessary, such as floristic surveys and environmental assessments.

We treated *Najas minor* and *N. oguraensis* as two distinct species, identified by a difference in number of anther locules. Les et al. (2015) treated them as one species, *N. minor*, because molecular phylogenetic analyses revealed that the same cpDNA haplotype and nrDNA ribotype were shared between them. However, a clear correlation between the number of anther locules and the size of the leaf epidermal cells confirms that an investigation of the biological status of these species is necessary. Further studies based on highly polymorphic markers, more precise morphological
examinations, cytological studies, and crossing experiments are needed to address the relationships of *N. minor* and *N. oguraensis*.

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**References**


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**APPENDIX I.** Specimen and voucher information for the taxa included in this study. DHS, leaves of dried herbarium specimens; DLS, dried leaf specimens.

*Najas minor* All.

