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Autor: Gil-Ad, Nir L.
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CHAPTER 2

MATERIALS AND METHODS

Herbarium Studies. – Initial data on distribution and phenology were compiled from herbarium specimens at MICH, US, NY, GH, VT, UC, NCU, DUKE, FSU, and MO (abbreviations follow HOLMGREN *& al.*, 1990). Specimens of subsection *Boreali-Americanae* bearing cleistogamous seeds were borrowed from GH, NDG, RSA, US and VT to supplement my collections. A total of 530 specimens of the subsection were studied in this research. In choosing specimens for study, emphasis was given to the quality of the specimens, i.e., the presence of informative organs, rather than to quantity. Pohl's solution (POHL, 1965) was applied to soften tissues in order to allow measurements on representative specimens.

Field Work. – Populations of the taxa in their natural habitats were studied during August 1989 in Maryland; May 1990 in Michigan, Pennsylvania, New York, New Jersey, Vermont, New Hampshire, Maine, and Massachusetts; during June 1990 in Modoc County, California; during March-May 1991 in Florida, Alabama, Mississippi, Louisiana, Texas, Nuevo-León, Oklahoma, Arkansas, Tennessee, Missouri, Kentucky, North Carolina, Virginia, and Maryland; during May 1993 in Michigan and northern California; during July 1993 in Maine; and during April 1994 in Tennessee. Observations were made on macromorphological variation, phenology, plant associates, substrate, and habitat specificity. A total of 287 collections of *Viola* (including some numbers consisting of series of specimens from populations of hybrids) were made. From most populations a few plants were pressed, and a number of living plants (usually 1-3) were transferred and cultivated in the greenhouse. The field work covered two types of sites: **a.** sites in which putative orthospecies were discovered; **b.** sites in which instances of hybridization or introgression were suspected.

Cultivation. – Plants collected in the wild were transplanted and grown in four inch pots on a shaded bench under natural day length in the research greenhouse at the Matthaei Botanical Gardens. Plants that grow in the wild on loamy soils tolerated well the commercial potting substrate Sunshine # 4 whose major components are Canadian dolomite, sphagnum peat, perlite, and a wetting agent. For such plants, a clump of soil was retained around the rhizomes and the roots, and was supplemented with Sunshine # 4 soil mixture to assist in anchoring the plants. Conversely, plants that thrive on specialized substrates such as sandy soil were grown only in the substrate taken from their native niche. Such plants do not tolerate the retention of water in commercial potting mixtures. Most plants were cultivated up to three years in the greenhouse. *Viola* requires vernalization in order to produce chasmogamous flowers (EVANS, 1956). Accordingly, the plants were placed in a cold frame in late November. Plants from southern latitudes were covered with mulch in the coldframe and transferred back into the greenhouse in February, and plants from northern latitudes were transferred back into the greenhouse in April.

Growing the plants in the greenhouse has made possible observation of the plants in both the chasmogamous and cleistogamous phases and at comparable developmental stages, and allowed observation of developmental characters, such as heterophylly. Observations were made on macromorphological variation, with a focus on reproductive characters. Characters that are not readily available on herbarium specimens were especially noted. These included: the color of the chasmogamous flowers, the shape of the petal trichomes, the shape and color pattern of the capsules, and the location and habit of the cleistogamous flowers.

Both chasmogamous and cleistogamous seeds are forcibly ejected up to five meters from the capsules of the taxa of subsection *Boreali-Americanae* (BEATTIE & LYONS, 1975). In order to prevent seed scattering, and the establishment of seedlings in wrong pots, cleistogamous capsules were covered prior to their maturation with aluminum foil, and seeds were harvested following

the dehiscence of the capsules. This technique has allowed monitoring the release of seeds, and enabled exact matching of seeds to their respective mother plants. Seeds were harvested from most of the plants, and are deposited at MICH.

The Value of Existing Herbarium Specimens of Subsection *Boreali-Americanae*. – During the preliminary stages of this study I discovered that in spite of the large number of herbarium specimens of *Boreali-Americanae* taxa that are deposited in the major North American herbaria, many are missing important characters, and are not very helpful, except for some of the information provided on the labels, such as locality, phenology, associates, and substrate. On many specimens the plants, especially the chasmogamous flowers, are imbedded in glue on the sheet. Consequently, careful examination of characters, such as reproductive characters, blade shape, and degree of division of divided blades, is not possible unless the integrity of the specimen is disrupted. Most of the specimens available in herbaria consist of plants that were sampled at the chasmogamous phase, and many of these specimens might be hybrids or introgressants. These specimens would not be helpful unless we devise means to identify putative hybrids and hybrid derivatives using the characters available on them. Unfortunately, macromorphological characters of chasmogamous plants are not adequate for the identification of hybrids.

When specimens of cleistogamous plants are available, their capsules are often opened and the pigmentation of the capsules is often faded or absent. In addition these specimens often lack seeds, or carry immature seeds whose pigmentation and micromorphology are incomplete or distorted. Mature seeds provide micromorphological characters that are helpful in distinguishing between orthospecies and nothospecies in subsection *Boreali-Americanae* (GIL-AD, 1995). Other important macromorphological characters that are difficult to depict from cleistogamous specimens are the color patterns of the capsules, the shape of the capsules, and the habit of the peduncles of the cleistogamous flowers.

I hope that future collectors would take the extra effort to re-sample populations from which they collected chasmogamous plants, or would cultivate plants transplanted from the same population from which the chasmogamous specimens were collected.

Preparation of Herbarium Specimens. – In order to overcome these problems, plants collected in this study were carefully prepared prior to pressing. The plants were stored and carried in plastic bags, and were prepared for pressing upon return from the field. The petals of the chasmogamous flowers and representative blades were spread and stabilized by placing over them paper strips that were taped to the newspaper. In a few cases additional flowers were harvested in the greenhouse. The use of a hot air drier was found to destroy the pigments in flowers, capsules and foliage, thus substantially reducing the quality of specimens. In addition, capsules on cleistogamous plants open prematurely upon heat-drying, and release immature, and in some cases, damaged seeds. Most of the specimens were pressed and air dried at ambient temperatures. The plants were mounted onto the sheet without imbedding them with glue. Rather, they were anchored to the sheet with paper strips. When available, a few extra plants were placed in envelopes attached to the specimens without any mounting. Capsules were studied on live plants, and representative capsules and seeds were photographed (see plates *in* GIL-AD, 1995). Seeds were harvested from live plants in the greenhouse, and were not exposed to any drying or pressing procedures. Representative plants at the cleistogamous phase were sampled and pressed to supplement the chasmogamous plants.

Blade Measurements. – The blade length/width ratio and apical angle were measured on the largest leaves of plants at chasmogamous anthesis, unless indicated otherwise. These blade parameters are illustrated in Fig. 1. Maximum blade length (L) was measured by the distance from the apex to the lowest margin of the basal lobe. In divided blades possessing lobes oriented downward an imaginary line connecting the apexes of the lobes was used to determine the lowest reference point for the length. Maximum blade width (W) was measured at the widest portion of the blade by the horizontal distance between the blade margins, or the apex of the widest horizontal lobe in divided blades. Apical angle (AA) was measured between the two lines extending from the apex and delimiting the margins of the blade, thereby depicting the shape of the apex.

In divided blades the apical angle was measured on the middle segment or lobe. The angle measured in this research differs from the angle measured by RUSSELL (1952, 1956b) and MCKINNEY (1992). They measured the angle of divergence from the horizontal (a line perpendicular to the midrib) of the apical margin of the blade, and designated it as apical angle.

Flower measurements. – The length of the spurred petal (AC) was measured from the edge of the spur base to the edge of the apex of the spurred petal. The length of the spur (AB) was measured from the edge of the spur base to the point of attachment of the nearest auricle (Fig. 2). The other measurements made were width measurements of the spurred petal, the lower lateral petals, and the upper lateral petals. The lengths of the upper and lower lateral petals were not measured since their measurements would have required taking the flowers apart, and thereby damaging the material.

Determination of Corolla Color. – The color of petals was compared in the field or in the greenhouse to color chips of the R.H.S. COLOUR CHART (1966). The chart was designed for horticultural taxa, and cannot be expected to include the full range of biological colors (TUCKER & *al.*, 1991). However, since it provides a better coverage of the variation in color and hue found in *Viola* than the ISCC-NBS Centroid Color Charts, it was selected for the determination of the color of the petals. The chips used for *Viola* belonged to the violet-blue and violet groups. Each chip in a group is numbered and divided into four blocks labeled A through D. When no exact match was found, the closest hue was designated. The major disadvantage of the R.H.S. Colour Chart is that it does not provide value and chroma or sometimes hue for each color chip (HUSE & KELLY, 1984). One has to consult the chart in order to get a good perception of the color denoted by a number and a letter. The color name and R.H.S. number listed in the description for each species (Chapter 6) were summarized from determinations of color from plants observed in different geographical areas (when available). Those plants were later determined as orthospecies using the seed micromorphology as one of the primary criteria. The color determinations should not be regarded as absolute since the color of petals may change under environmental stress and modifications in the pH of the substrate. Hybridization and introgression introduce an additional

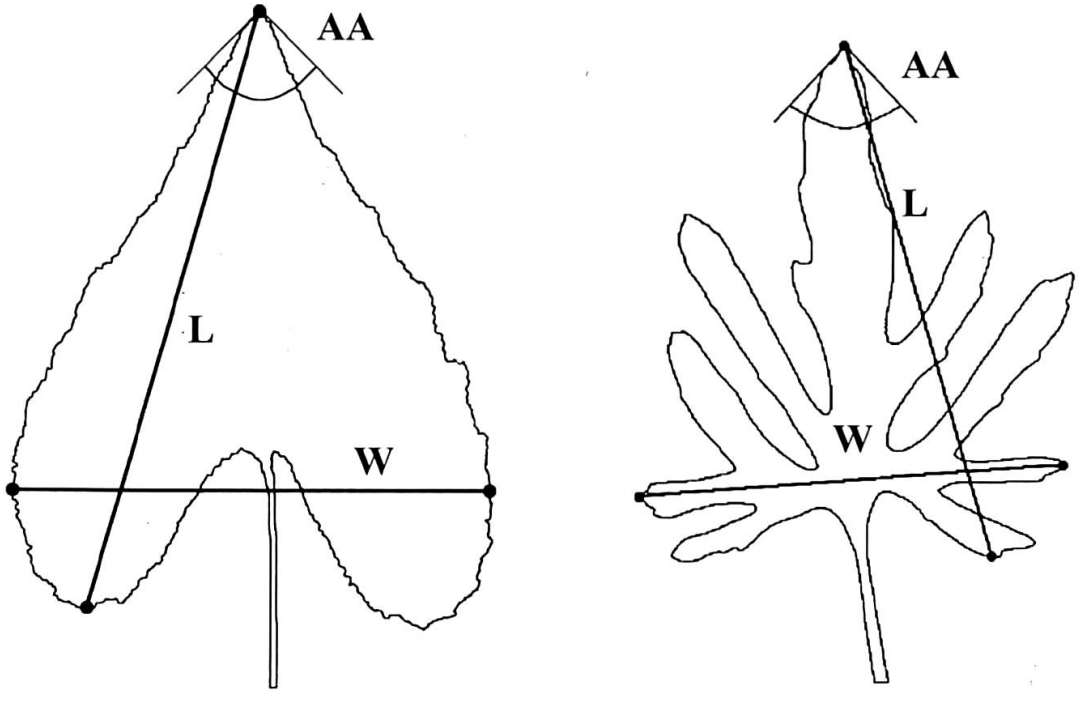

Fig. 1. – Outlines of representative undivided and divided blades, and the parameters measured to characterize the blades. Abbreviations: **L** = maximum length; **W** = maximum width; **AA** = apical angle.

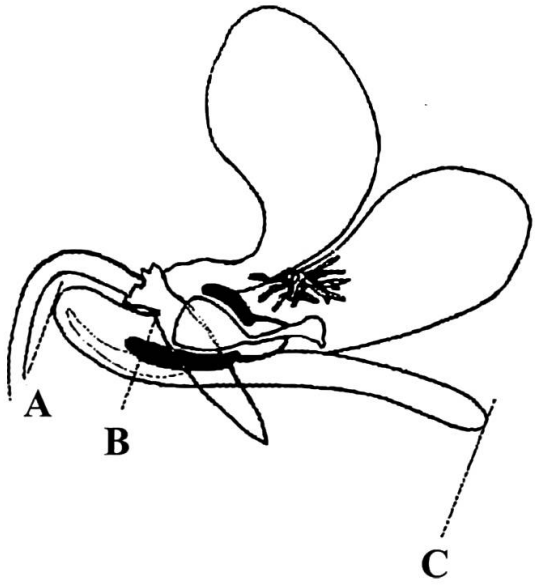

Fig. 2. – A longitudinal section in a representative flower of *Viola* subsection *Boreali-Americanae* (with some of the organs removed), and the parameters measured to characterize the flower: **A-B** = spur length, **A-C** = length of the spurred petal.

variable. Therefore, the color of the petals should be used as an accessory character, and not as a primary character for the delimitation of taxa.

Measurements of Seed Dimensions. – Seeds of the taxa of subsection *Boreali-Americanae* that are produced by chasmogamous and cleistogamous flowers show no significant difference in morphological characters (GIL-AD, pers. observations). In most cases measurements were made on cleistogamous seeds since they were more readily available. Seed length, seed width, and caruncle length were measured using a dissecting microscope equipped with an ocular micrometer (precision 0.1 mm). At least ten seeds were measured per specimen by sampling the range of sizes per sample. When seeds were obtained from live plants, each sample of ten seeds was taken from a different capsule. Ranges for each parameter per specimen were constructed, compared and combined with ranges obtained from other specimens of the same taxon. Caruncle width was found to be too variable even among the seeds of one plant, and caruncles exhibited shape and width variation that would not allow the employment of consistent reference points for measurement.

Measurements of Seed Weight. – A Mettler H-20 analytical balance was employed to determine seed weight. At least ten seeds were weighed per each specimen or live plant, and mean weight was calculated. When enough seeds were available on a specimen, up to ten measurements were conducted, and a range was constructed for the calculated means.

Determination of Seed Color. – The overall seed color of mature *Viola* seeds is comprised of the color of the primary sculpture (background color) and the color of the secondary sculpture (overlaid color). Intraspecific variation in seed color is not common, but may occur when the color of one of the components is more dominant. The overall seed color was determined by using the ISCC-NBS Centroid Color Charts (KELLY, 1965). Each color block is labeled by a number, and a descriptive name. Each color block name consists of the name of a neutral or color hue combined with one or more modifiers with the color hue themselves being used as modifiers, e.g., 72 dark orange yellow (MCKNIGHT, 1977). Five mature seeds were sampled from each specimen. Determination of color was done under a full spectrum light generated by a CHROMALUX ®100W full spectrum bulb. The overall color of the seed surface was compared to the color blocks in the charts, and a matching color was identified. In cases where no exact match could be found, the closest color block names were used to characterize and describe the color.

Ecological and Developmental Observations. – A number of observations on the ecology and development of the taxa have been made throughout this study. Due to limitations of time and resources they were not pursued thoroughly. Therefore, they should be regarded as preliminary observations that require additional studies.

The interested reader is advised to refer to the following publications that cover related aspects of *Viola* biology and ecology: biology of *Viola fimbriatula* in a natural disturbance (COOK & LYONS, 1983), cleistogamy (MADGE, 1929; WEST, 1930; THÉRON, 1939; HOLDSWORTH, 1966; BASKIN & BASKIN, 1975b; MAYERS & LORD, 1983a, b), environmental effects (CURTIS, 1984; CURTIS & KINCAID, 1984; YOST, 1987), floral biology and evolution (BEATTIE, 1969, 1974), pollination and gene flow (BEATTIE, 1971, 1976, 1978), seed dispersal (GATES, 1943; BEATTIE & LYONS, 1975; CULVER & BEATTIE, 1978, 1980; BÜLOW-OLSEN, 1984; OHKAWARA & HIGASHSI, 1994), seed dynamics and longevity of *Viola fimbriatula* (ANDERSON, 1983), photoperiod, vernalization and phenology (BORGSTRÖM, 1939; ALLARD & GARNER, 1940; CHOUARD, 1948; EVANS, 1956; RUSSELL, 1960), population biology (BEATTIE, 1979; SOLBRIG *& al.*, 1980; SOLBRIG, 1981; NEWELL *& al.*, 1981; YOST, 1984).

Determination of Soil Type. – Soil samples (ca. ten cubic centimeters) were removed from the area surrounding the roots of *Gil-ad 389* (MICH): *Viola nuevo-leonensis*, and *Gil-ad 460* (MICH): *V. brittoniana*, and analyzed by Michigan State University Soil Testing Laboratory. Results are reported in the discussion of these species. The substrate for the other taxa was determined by observations in the field and from reports in the literature.

Scanning Electron Microscopy of Seeds and Petal Trichomes. – Details of the sampling procedures of seeds and petal trichomes and the SEM procedures employed to examine them are provided in GIL-AD (1995) and GIL-AD (in press).