LEAF-PRINT ANALYSES: AN ECOLOGICALLY FRIENDLY METHODOLOGY FOR PLANT IDENTIFICATION

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ABSTRACT

Because the taxonomy of *Stanhopea* (Orchidaceae) has been established exclusively on analyses of the flower, field identification is complicated by the fugacious habit. As an aid to species confirmation, a system is offered that is ecologically friendly, economical, and statistically based. Clinical use of this system suggests it may also have value in determining the degree to which natural and man-made hybrids are related to each species-parent.

RESUMEN

Debido a que la taxonomía de *Stanhopea* (Orchidaceae) ha sido establecida exclusivamente en base al análisis de la flor, la identificación en el campo es complicada debido a su fugacidad. Como ayuda para la identificación de esta especie, se ofrece un sistema que es respetuoso ecológicamente, económico, y con base estadística. El uso clínico de este sistema sugiere que posiblemente tenga valor en la determinación del grado en el que los híbridos naturales y los obtenidos por el hombre están relacionados con cada parental.

INTRODUCTION

The genus *Stanhopea* was named in honor of Sir Philip Henry, the 4th Earl of Stanhope (1791–1855), president of the London Medico-Botanical Society from 1829–1837. Estimates of its size are numerous with old published estimates often repeated without reference to recent sources: Hawkes (1965: 8–25 or more); Hamer (1974: ca. 20); Arditti (1992: approximately 50); Dressler (1993: 55); and Bechtel et al. (1992: ca. 25). Jenny (1993) offers "about 52 species, two varieties (subspecies) and six natural hybrids." A search of the literature coupled with clinical research suggests that combining the Dressler and Jenny estimates offers the most accurate estimate for the size of *Stanhopea*.

The genus is known from Mexico, throughout Central America, eastward across northern South America, and south-southwest into Bolivia, Ecuador, and Peru. Its northernmost invasion is reported by Kennedy (1974) from western Mexico, at a latitude farther north than San Antonio, Texas

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while its southernmost reach is from the area of São Paulo, Brazil (Pabst & Dungs 1977).

Floral keys have been offered for regional areas, with most concentrating on Mexico. A key by Williams (1951) was followed by publications by Ames and Correll (1952, 1953), and a key to the Mexican members by Dodson (1963). Kennedy (1975), working with Dodson's more recent taxonomy (1975), published a hierarchy of the genus *Stanhopea* in Mexico, and more recently Williams and Whitten (1988) offer a key to the *Stanhopea* species of Panama. However, a comprehensive well illustrated treatment of the genus has yet to be offered and, in fact, the genus has compratively ignored because its flowers are generally not long lasting. Curtis (1910) cites *Stanhopea* flowers as large and very attractive, but notes that "they are short-lived and cannot be used in floral decorations, hence cannot be considered first class." Stanhopeas have thus been relegated to the domain of orchid aficionados with the desire and space to maintain them, and as an occasional study topic by orchid botanists.

Species identification is necessary for government support of conservation. Lawmakers bluntly want to know the specific identity of the organism targeted for government conservation support. In addition, compared with other national priorities, funds for conservation are in short supply in the United States and are even less available in most of the countries situated in tropical areas where much of the world's orchid flora is abundant. Thus, working against their preservation are explicit and implicit costs attendant to identifying orchids in their habitats.

New problems arise as field collections are to be made of material to be used in the identification process. Governments often take a dim view of removing plants or plant parts, and—obviously—any plant that's collected, pressed and dried will never again set seed in its natural habitat. "The removal of even a "window" of lamina, as outlined by Cutler (1978) and utilized by Stern and Morris (1992) damages the plant (albeit minutely), opens the door to local political restrictions regarding the removal of plant material from the habitat, and requires detailed laboratory dissection and staining procedures. In general, as procedures become more complex, equipment costs increase and the likelihood of artifact introduction likewise increases. Adding to the problem is that some of these orchids flower for only very short periods during the year. *Stanhopea* flowers last only a few days so one needs to be at exactly the right place at the right time or to be able to identify members of this genus when they're not in flower. However, most orchid taxonomy is based on analyses of the flower. Indeed, Curry et al. (1988) state "the taxonomy of *Stanhopea* species rests *exclusively* (italics ours) on the morphology of the flower, changes which have apparently been influenced by the pollinators."

Thus, although problems remain with trying to correctly identify plants, this approach attempts to be bounded by parameters which clearly meet local economic, political, and botanical benchmarks. Strict requirements have been self-imposed for this study, and the identification system must meet all of the following tests:

1. It must require minimal material in the field and only that equipment in-house or easily obtainable by the laboratory of a foreign university.
2. The field work and laboratory work should be able to be carried out by any properly trained secondary science student or university undergraduate student of average ability.
3. The identification protocol should be "ecologically friendly," that is, it should not damage the plant being tested, nor should it require any part of the plant to be removed from its habitat. Briefly, it should be possible to obtain a print-sample from the plant, tag both the print-sample and the plant for field identification purposes; carrying out the identification protocol with minimal disturbance of the plant in its habitat.
4. The system must be simple and the methodology inherently economical. An identification protocol requiring extensive, expensive, detailed procedures, material, and equipment is patently undesirable in countries where economic pressures are particularly acute and government, private agencies, and individuals are all hard-pressed to fund conservation-oriented work.
5. Identification confirmations must be objective, not subjective. Identification predictions should be supported by statistical methodology not only by the personal opinion of individual A or B.
6. Lastly, and probably the greatest "acid test" is that the system must reasonably work in the "real-world" and constructively contribute to the body of botanical knowledge. What is sought is not merely a simplistic method of helping provide correct plant identifications, but one that contributes to a greater understanding of species and genera, and assists in their conservation and a more enlightened awareness and appreciation of their economic and ecological value by the general populace.

The genus *Stanhopea* was selected for several reasons. In nature, members flower only for a few days, resulting in field identifications being a matter of seeing the plant at the right time, and—as one of the genera suggested by Dr. Carl Withner—it appeared that a sufficient number of study specimens could be secured on a limited budget. The genus offers a particularly interesting challenge because, despite being represented throughout the tropics of the New World, it will not normally be encountered when in flower, thus presenting a practical group for the investigation of an identification confirmation system using means other than floral analyses.

The use of fingerprinting by law enforcement agencies depends, in part, on having a sufficiently extensive file of known prints against which an "unknown" may be checked against the three general groups of arches, loops, and whorls (U.S. Dept. Justice 1984). Among other advantages, this system is non-invasive; doing no damage to the individual being printed. Although the print match may be attempted by mating prints from an object directly with those of an individual, the system can be effective by comparing prints from an object with those from an extensive file of known

subjects. With this background from law enforcement work, particular note was taken of "leaf fingerprinting" used by students at the Universidad Autónoma de Nuevo León, the specific methodology of which was said by the professor to be unpublished work by him. However, just prior to returning the galley proofs of this manuscript, a publication by Petroski referencing one by E.M. Stoddard, was received from Dr. John Beckner of the Marie Selby Orchid Identification Center at Sarasota, Florida. Petroski (1965), outlines a similar method of leafprinting of orchid leaves using cellulose acetate (clear fingernail polish), with the resulting dried cellulose acetate film removed by forceps and dry-mounted on a microscope slide. This work's basic fingerprinting technique does not greatly differ from that of Petroski (1965) and Stoddard (1965), although the statistical analyses of the cell measurements developed by one of our number (Ferry) offers a new approach, objective in nature, to the identification of species. In addition, Stoddard's work with alfalfa, chrysanthemums, and marigolds infers the usefulness of this system to other plant families.

METHODS AND MATERIALS

Clean white styrofoam "popcorn" is dissolved in xylol until the liquid is about the consistancy of warm syrup. This is applied to a clean leaf surface over an area of $\pm 2 \times 5$ cm, drying in two or three minutes. A short strip of clear transparent tape is pressed evenly and firmly over the film, but not with enough pressure to damage the leaf. The tape is peeled from the leaf, gently pressed onto a glass slide, and examined with a compound microscope. If it is desired to retain the slide permanently, a thin glass coverslip may be applied with its longitudinal edges taped to ensure holding the leaf print flat.

The slide should be marked as to which leaf surface, adaxial or abaxial, was printed. Using a felt writer or other marking pen, a small "H" or an "E" (haz: Spanish for adaxial, or envéz for the abaxial or underside of the leaf) is normally marked. The letter is followed by six digits to indicate the date, always as day-month-year (e.g. H020496/7 = Haz; 02 April, 1996/ the seventh specimen done on that date). This writing is small and done where it can be removed when the slide's permanent label is placed. In the field, the slide is now placed in a slide box or small envelope, and a small plant tag gently tied to the plant with the same set of numbers (H&E020496/7) penciled on both sides or imprinted with a stylus. It is imperative that the location be clearly stated on either the envelope or a card within the envelope for relocating the plant at a later date!

In the laboratory, the slide is photographed at 80X magnification. The microscope used in this research is a Microscoptics compound microscope with a trinocular head on which is mounted a Nikon HFM photo system.

An indexing lens inserted in the field lens assembly of the photosystem prints index marks on each photomicrograph enabling measurements to be taken from the print. A Reichert-Jung micrometer slide of 2 mm divided into units of 0.01 mm is used to establish the lens correction factor for each magnification capability of the microscope.

Black and white or color film may be used, and processed privately or commercially. In this study, Kodacolor film was used to make 10 × 15 cm (4 × 6 inches) prints. For color transparencies, Kodak Tungsten film has been the film of choice. From the photographs, 25 each of adaxial cells, abaxial cells, guard cells (both as a unit) and subsidiary cells are measured using a set of calipers to measure average lengths and widths. The number of trichomes (adaxially and abaxially) shown on each photomicrograph is noted on the specimen's data sheet, as are the number of stomata.

A Macintosh SE/30 computer with 5 mb of RAM and 80 mb of internal memory was initially employed, augmented later by a Power Tower Pro 225 computer with 128 mb ram and 2 gb of internal memory. A Microsoft Excel spreadsheet combined each raw measurement with the lens correction factor, entering measurements on the data sheet in microns (μ) and combining them to present the total, mean, and standard deviation for each set of measurements. The statistical treatment used was an analysis of variance (ANOVA) with $\rho = .05$. The number of cells per square millimeter was computed and printed on the specimen's data sheet. Totals from each individual data sheet are linked to provide a combined worksheet from which data totals are analyzed, and individual and groups of Gaussian curves can be obtained on printouts.

RESULTS

Data have been collected from fifty *Stanhopea* specimens, 32 of which are different species (multiple samples of some), and two man-made primary hybrids. Data have also been collected from plants of *Govenia utriculata* (Sw.) Lindley, *Govenia superba* (Llave & Lexara) Lindley ex Loddiges, *Malaxis corymbosa* (S. Watson) Kuntze, and *Malaxis macrostachya* (Lexara) Kuntze in the Sierra Madre range southwest of Monterrey, Mexico. Subsequent checking indicates no damage done to any field or greenhouse plant from which leaf-prints have been taken.

A *Stanhopea* plant received as an "unknown" on 29 April, 1996 was numbered C26 and data taken from it were compared with that from known plants. Comparisons of the mean of its adaxial cell areas with known species ($r = .05$) indicated it as *S. tigrina*. On 20 June, 1996 another unknown arrived and was tagged C33. The data inferred that it too was *S. tigrina*. On 08 July plant C26 flowered, confirming the prediction made on the basis of the statistical data from the leaf print. This provided the first example of

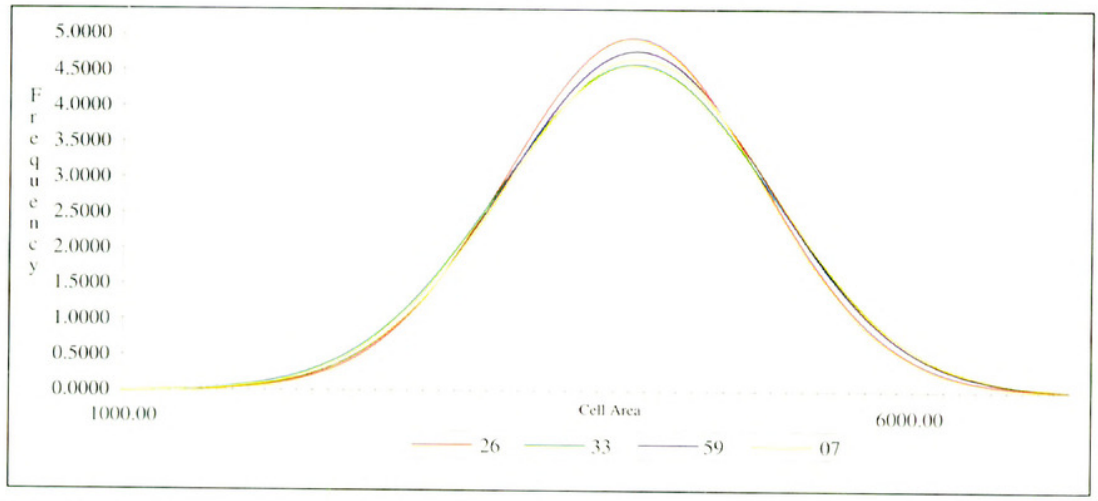

FIG. 1. Gaussian curves of adaxial cell areas of *S. tigrina* specimens C26, C33, C59, and C07.

the successful prediction of the plant's identity by use of this methodology prior to seeing it in flower. A few days later, despite the destruction of plant C33 by a resident macaw, analysis of the remains of a not-fully-opened flower confirmed that its predicted identification had also been correct. Gaussian curves are presented for plants C26 and C33 and the two confirmed *S. tigrina* plants (C07 and C59) in Figure 1.

Although the normal curve for specimen C26 was more leptokurtic (higher "crested") than the others, the adaxial means of all specimens did not significantly differ (ANOVA, $\rho = .05$). Amplified data for the four *Stanhopea tigrina* specimens is shown in Table 1.

Stanhopea Chocolate Chips = *S. tigrina* × *panamensis* by D. Pulley, 1991 (Fisher 1994). Data from specimen C56, Chocolate Chips 'Lindt' was compared with that from C07 *S. tigrina*, C59 *S. tigrina* 'Glory of Mexico,' and C58 *S. panamensis*, all of which were supplied by Dr. Douglas Pulley of Los Gatos, California. While the exact *S. tigrina* parent of this hybrid was unknown by this worker, all plants had been received from the same grower-hybridizer, and the assumption was that one of the two *S. tigrina* plants was one parent and the *S. panamensis* plant the other. Therefore both *S. tigrina* plants are included in the chart of the Gaussian curves and Table 2 gives a summary of the data for the four specimens.

DISCUSSION

Analysis of the adaxial cell area means of the hybrid and parents (Table 2) infers that S. Chocolate Chips 'Lindt' is vegetatively more closely allied with the *S. tigrina* parent than with *S. panamensis*. This is borne out pictorially by the position and shape of the curves (Figure 2), and abaxial epidermal cells indicate a similar relationship. However, correlation attempts using stomata guard and subsidiary cells have been inconsistant in and between

Table 1. Statistical data: Four specimens of *S. tigrina* (C07, C26, C33, & C59). Specimen C59 is *S. tigrina* 'Glory of Mexico.' Note that areas and numbers of cells are means derived from data collections. Ad: adaxial; StdDev: standard deviation; Ab: abaxial; Aad/Aab: area, adaxial/area, abaxial; GCl/w: guard cells length/width; SSarea: area, subsidiary cells; SSl/w: subsidiary cells length/width.

C#	Ad Area	Ad#Cells	AdStdDev	AbArea	Ab#Cells	AbStdDev	Aad/Aab	GCArea	GCl/w	SSarea	SSl/w
07	4259	234.79	852.13	2857.66	349.94	700.88	1.49	919.92	1.42	2126.08	1.50
26	4220	236.94	805.00	2856.17	320.12	734.29	1.48	1128.68	1.25	2159.94	1.30
33	4234	236.21	869.12	2816.88	355.00	815.48	1.50	1537.75	1.34	1483.57	1.25
59	4245	235.57	836.57	2917.18	342.80	639.82	1.46	1817.58	1.48	3745.29	1.25

Table 2. Statistical data: C07 *S. tigrina*, C59 *S. tigrina* 'Glory of Mexico,' C56 S. Chocolate Chips 'Lindt,' & C58 *S. panamensis*. Ad: adaxial; StdDev: standard deviation; Ab: abaxial; Aad/Aab: area, adaxial/area, abaxial; GCl/w: guard cells length/width; SSarea: area, subsidiary cells; SSl/w: subsidiary cells length/width.

C#	Ad Area	Ad#Cells	AdStdDev	AbArea	Ab#Cells	AbStdDev	Aad/Aab	GCArea	GCl/w	SSarea	SSl/w
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59	4245	235.57	836.57	2917.18	342.80	639.82	1.46	1817.58	1.48	3745.29	1.25
56	4095	244.19	850.31	2828.01	353.61	622.69	1.45	1566.14	1.30	3477.2	1.39
58	3724	268.50	822.46	2657.20	376.34	392.69	1.40	1541.51	1.32	1484.26	1.58

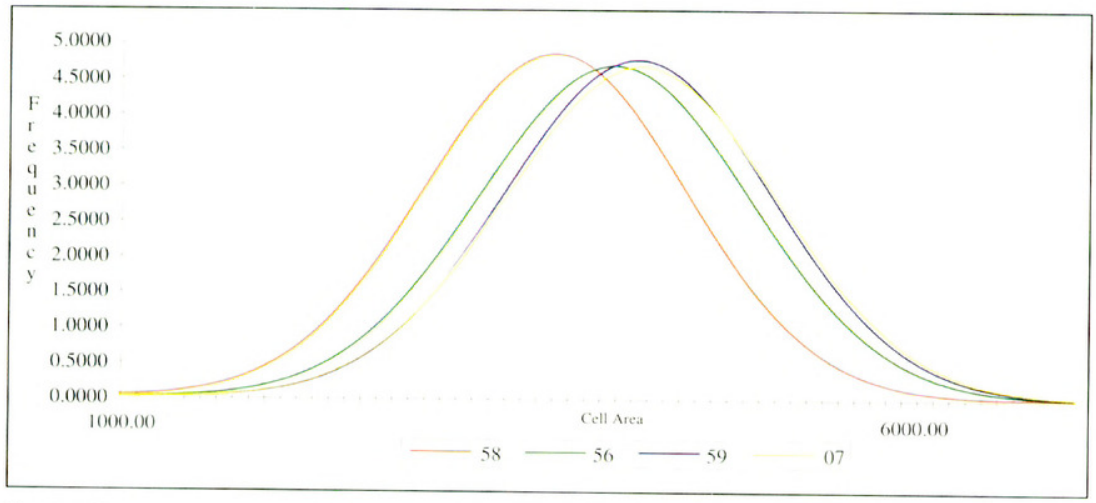

FIG 2. Gaussian curves of adaxial cell areas of C58 *S. panamensis*, C56 S. Chocolate Chips 'Lindt,' and *S. tigrina* specimens C59, and C07.

species as well as between species and their hybrids. More trials linking leaf cell data with proven floral qualities are indicated. If parent-offspring curves of adaxial cell areas or areas of other external organs can be correlated with specific floral qualities sought by the hybridizer, these results could be of economic value as a predictor of floral characteristics while still in the seedling stage and could afford the grower improved quality control over seedling crops. With regard to taxonomic avenues, it is hypothesized that the statistical relationship established by DNA sequencing will not significantly differ from that arrived at by this methodology. If this proves to be the case with botanical specimens, a vast array of possibilities may be applicable to other life forms.

The identification of these unknowns offers encouragement for the greater use of this leaf-print-statistical analysis methodology for confirming plant identities in nature, and preliminary field studies lend support to the use of this methodology. Adapted for field use, population surveys appear to be possible without disrupting the ecosystem, while affording humans the option of hand-pollinating specifically identified field plants, thus assisting in reviving a low plant population without disturbing its members.

In the field, this system could be of use in confirming the identity of both species and hybrids and provide the number of each in specific areas. Grant's work (1981) with the genus *Gilia* in California provides strong evidence that—with sufficient time and the invasion and adaptation of a species into new habitats—a species can vary sufficiently over its range to provide new fixed gene combinations resulting in a new species. Grant's basic data was originally published in 1971 with the 1981 edition providing refinements, corrections, additional data, and preliminary results of unpublished clinical work with specimens of *S. saccata* and *S. radiosa* ap-

pear to provide confirmation for his work. In light of the work of Dodson (1963) and of Kennedy (1975), it is suggested that field identification work using this statistical methodology over the range of the *Stanhopea radiosa-saccata* complex would provide useful data for clarification of this speciation phenomenon and assist in establishing specific points of variation at precise locations over the geographical range of the two species.

In conclusion, this is an objective approach to plant identification confirmation relying on data capable of statistical verification. In outlining the six requirements listed earlier in this paper, the attempt has been to detail a system workable within the realities of governmental attitudes, and the conservation desires of the scientist; yet one capable of being easily understood and supported politically and economically by government agencies and local people within the countries concerned.

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