# Molecular and Morphological Investigation of Ornamental Liriopogons

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ABSTRACT. Eighteen commercial taxa in genera Liriope and Ophiopogon were evaluated genetically and morphologically. Three hundred and forty-four Amplified Fragment Length Polymorphism (AFLP) markers were identified and used in the analysis. Relatedness among taxa was estimated by analyzing the AFLP data using the Dice coefficient of similarity and UPGMA methods. The molecular analysis revealed relatively no genetic differentiation among the taxa ascribed to their respective genera, Liriope and Ophiopogon, based on morphological assessment of floral structure. Greater than 95% of the total genetic variability present was attributed to within group effects using various grouping strategies among the taxa. Among group effects were correspondingly low and not significant ( $P \le 0.05$ ). Results suggest an indistinguishable genetic relationship may exist in the tribe Ophiopogonae between what is currently ascribed as two distinct genera.

Liriopogons (Convallariaceae tribe Ophiopogoneae Endl.) are the most important clonal ground covers sold by the nursery industry in the southeastern United States. The term liriopogon proposed by Skinner (1971) collectively represents ornamentals in the genera *Liriope* Lour. and *Ophiopogon* Ker-gawl. (aztecgrass, lilyturfs, monkeygrass, and mondograss) and denoted an underlying morphological similarity between the two genera. For example, the popular aztecgrass is referred to commonly as a *Liriope* sp., but in actuality it is identified taxonomically as an *Ophiopogon* sp. (Adams, 1989). This similarity has led to industry-wide misidentification of genera and putative clones possessing multiple trade names (Fantz, 1994).

The industry widely regards cultivars in *Liriope* as possessing evergreen grasslike leaves, erect flowers with a superior ovary (Adams, 1989), and berrylike, fleshy black fruit (Liberty Hyde Bailey Hortorium, 1976). Species in the genera Ophiopogon possess narrow, evergreen grasslike leaves, drooping flowers with an inferior ovary (Adams, 1989), and a blue berrylike fruit (Bailey and Bailey, 1976). Arecent taxonomic treatment of the Convallariaceae differentiated the genera based mostly on flower part insertion (Conran and Tamura, 1998). Flowers of *Liriope* are hypogynous with the petals inserted around the base of the gynoecium, and slightly zygomorphic (bilateral symmetry). Seeds are blackish. Flowers of Ophiopogon are perigynous, i.e., the petals are attached to a cuplike hypanthium which arises round the base of the gynoecium, and actinomorphic (radial symmetry) (Fig. 1). Seeds are blue. In contrast, Rudall et al. (2000) uses hemi-epigynous to describe Ophiopogon flowers. The genus Peliosanthes Andrews shares these later characteristics and represents a putative third genus in

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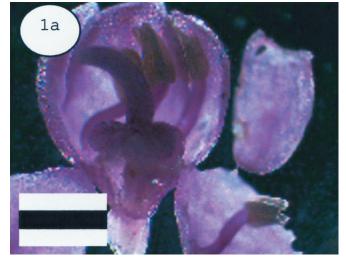




Fig. 1. Photomicrographs showing flower morphology of *Liriope* sp. and *Ophiopogon* sp.: (**top**) 'Royal Purple' (*Liriope* sp.) showing a hypogenous ovary insertion, (**bottom**) aztecgrass (*Ophiopogon* sp.) showing a perigynous ovary insertion. Bar = 1.0 mm.

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Ophiopogoneae. *Peliosanthes* spp. have a corolla (inner set of floral leaves) whereas *Ophiopogon* spp. do not. Further refinement to species, e.g., *Liriope spicata* Lour., is more daunting, requiring mostly cellular level characterization (Cutler, 1992). Cultivar identification is difficult and confounded by inconsistent naming.

Molecular characterization is an approach useful in assigning cultivars or clones to genera and identifying unique banding profiles capable of fingerprinting cultivars for definitive identification. To date, no reports exist in the literature using a molecular approach for these genera, notwithstanding a systematic investigation of the Convallariaceae by Rudall et al. (2000). Single accessions of the aforementioned genera were included in broader molecular and morphological analyses of Convallariaceae. Rudall et al. (2000) suggested that just *Liriope* and *Ophiopogon* belong in Ophiopogonae.

The objective of the present study was to use a combination of morphological descriptors and DNA fingerprinting to differentiate common industry taxa to the proper genera, to describe the extent of genetic similarities among these taxa, and to identify possible duplicate taxa.

#### **Materials and Methods**

PLANT MATERIAL. Clones of 12 common industry cultivars (Big Blue, Christmas Tree, Densiflora, Evergreen Giant, John Birch, King Felix, Monroe White, Royal Purple, Samantha, Silver Dragon, Silver Midget, and Variegata), three clones referred to by their common name (aztecgrass, black mondo, and mondograss), two clones referred to by their cultivar-group name (minimondo and variegated mondo), and one clone referred to by its species (L. spicata or spicata) were collected from nurserymen in Louisiana or from an in situ collection at Louisiana State University Agricultural Center, Burden Research Station, Baton Rouge, for inclusion in the study. The term taxa collectively represent cultivars, common names, cultivar-group names, and specie in this study. From June through August 2002, inflorescences were collected, preserved in FAA (ethanol 47.5 %, glacial acetic acid 5 %, formaldehyde 4 %, and water 43.5 %) solution for later microscopic characterization. Also, voucher specimens were prepared and deposited in the Louisiana State University herbarium.

**PLANT DNA EXTRACTION.** Young leaves were harvested, freezedried, and stored at -20 °C until needed. Total DNA was isolated from 100 mg of leaf tissue using the Genelute plant genome kit (Sigma-Aldrich Inc., St. Louis, Mo.).

AFLP ANALYSIS. Techniques for amplified fragment length polymorphism (AFLP) fingerprinting were adapted from those described by Vos et al. (1995). In total, 0.08  $\mu$ g of total genomic DNA from each sample was double-digested with 1.25 units each of a mixture of EcoRI and MseI (AFLP Core Reagent Kit, Gibco BRL, Gaithersburg, Md.) in 5× reaction buffer [50 mm ris-HCl (pH 7.5), 50 mm Mg-acetate, 250 mm K-acetate] in a final volume of 6.25  $\mu$ L. The reaction was incubated at 37 °C for 3 h. Then 6  $\mu$ L of a mixture containing EcoRI and MseI adapters [EcoRI/MseI adapters, 0.4 mm ATP, 10 mm Tris-HCl (pH 7.5), 10 mm Mg-acetate, 50 mm K-acetate, 0.25 units of T4 DNA ligase] were added and the reactions were incubated at 18 °C overnight. Samples were subsequently diluted 1:10 with TE buffer and stored at -20 °C.

The preselective PCR amplifications were performed in 13  $\mu$ L reaction volumes each of which contained 10  $\mu$ L preamplification primer mix, 1.5  $\mu$ L diluted adapter-ligated genomic DNA, 1.25 unit *Taq* DNA polymerase (Promega, Madison, Wis.) and 10× PCR buffer [100 mm Tris-HCl (pH 8.0), 15 mm MgCl<sub>2</sub>, 500 mm KCl].

Preselective PCR amplifications (28 cycles) were performed as follows; 30 s at 94 °C, 30 s at 56 °C, and 60 s at 72 °C.

The selective PCR amplifications were performed in 20  $\mu$ L reaction volumes each of which contained 2.5  $\mu$ L diluted (1:10) preamplified DNA template, 4.4  $\mu$ L MseI primer (6.7 ng/ $\mu$ L), dNTPs (200  $\mu$ M each), 0.4  $\mu$ L (0.5 pmol) of IRD700-label EcoRI primer (LI-COR, Lincoln, Neb.), and 1 unit of Taq polymerase, in 10× PCR buffer [100 mM Tris-HCl (pH 8.0), 15 mM MgCl<sub>2</sub>, 500 mM KCl]. The conditions for selective PCR amplifications were as follows; 12 cycles of 30 s denaturation at 94 °C, 30 s at 65 °C (-0.7 °C/cycle) until reaching an annealing temperature of 56 °C, and extension for 60 s at 72 °C. This was followed by an additional 28 cycles of 30 s at 94 °C, 30 s at 56 °C, and 60 s at 72 °C.

All PCR reactions were conducted in a thermal cycler (GeneAmp PCR System 9600; Perkin-Elmer Corp., Foster City, Calif.). PCR amplification fragments were separated by electrophoresis in 6.5% polyacrylamide using a DNA sequencer (Long ReadIRTM 4200L-1; LI-COR protocols, http://www.bio.licor.com/pubs/biopub/htm; LI-COR, Lincoln, Nebr.).

DATA ANALYSIS. Gel images were recorded during electrophoresis and analyzed with Gene ImagIR software (LI-COR, version 3.55). AFLP markers were scored for the presence (1) or absence (0) of bands. Data were analyzed (NTSYS-PC software version 2.0; Rohlf, 1993) in two steps. Similarity values were calculated using Dice's coefficients of similarity (Sneath and Sokal, 1973). A dendrogram was then constructed based on the matrix of the similarities using the Unweighted Paired Group Method (UPGMA) (Sneath and Sokal, 1973). An Analysis of Molecular Variance (AMOVA) was also used to estimate variance attributed to among and within genotypic groups (Huff et al., 1993). Genetic distances for the AMOVA analysis were estimated using the Euclidean metric distance of Excoffier et al. (1992) defined as  $E = n [1 - 2n_{xy}/2n]$ , where  $2n_{xy}$  is the number of markers shared by two individuals, and n is the total number of polymorphic markers. The AMOVA analysis was performed using WINAMOVA 1.55 software (Excoffier et al., 1992).

#### **Results**

Morphological characterization. Hypogynous flowers were found on 'Big Blue', 'Christmas Tree', 'Densiflora', 'Evergreen Giant', 'John Birch', 'King Felix', 'Monroe White', 'Royal Purple', 'Samantha', 'Silver Dragon', 'Silver Midget', spicata, and 'Variegata', affirming their inclusion in the genus *Liriope* (Fig. 1a). Perigynous or hemi-epigynous flowers were found on aztecgrass and black mondo, affirming their inclusion in *Ophiopogon* (Fig. 1b). Three of the 18 cultivars seldom produced flowers. Undeveloped flowers of variegated mondo were found on one plant and are best described as hemi-epigynous. No flowers were found on minimondo; mondograss had some undeveloped inflorescences, but no flowers were found during our evaluation period.

**MOLECULAR CHARACTERIZATION.** A total of 344 polymorphic AFLP markers (65 bp to 160 bp) were scored from four primer combinations (E-AAG/M-CTT, E-AAG/M-CTG, E-AAG/M-CAG, E-AAG/M-CTA); discrimination between AFLP markers was poor outside of this size range. The number of AFLP markers per genotype ranged from 64 ('Christmas Tree') to 94 ('Samantha' and 'Silver Dragon') with a mean of 78.

The 344 polymorphic AFLP markers were sufficient to differentiate all 18 taxa (Fig. 2). Similarity values ranged from 0.23 to 0.57 (data not shown). The highest level of similarity (0.57) was between two groups of *Liriope* spp. cultivars, Big Blue/Silver Dragon and Samantha/Variegata, and the lowest was a group of

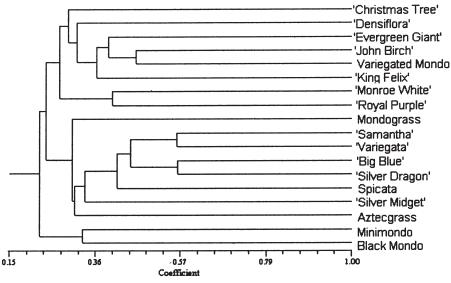


Fig. 2. UPGMA based dendrogram of 18 *Liriope* and *Ophiopogon* taxa. The numerical scale indicates increasing genetic similarity.

two mondograsses (black mondo and minimondo) and all other taxa (0.23), including other mondograsses.

The AFLP marker-based UPGMA dendrogram (Fig. 2) depicts the relationships between individual plants. The goodness-of-fit was good(r=0.83). *Liriope* taxa mostly showed similarity. *Ophiopogon* taxa were poorly grouped, exemplified by variegated mondo.

A number of different groupings were analyzed using AMOVA, e.g., *Liriope* taxa with and without variegated mondo, and variance components were estimated. The majority of the total genetic variability present was attributed to within group effects and was estimated to be >95%. The among group variance component accounted for <5.0% of the total variability using various grouping strategies, but none showed significance ( $P \le 0.05$ ).

## Discussion

Floral structure mostly affirmed previous industry perceptions that 'Big Blue', 'Christmas Tree', 'Densiflora', 'Evergreen Giant', 'John Birch', 'King Felix', 'Monroe White', 'Royal Purple', 'Samantha', 'Silver Dragon', 'Silver Midget', spicata, and 'Variegata' belong to the genus *Liriope* based on the Convallariaceae generic key in Conran and Tamura (1998) (Fig. 1a). Flowers were less common on putative *Ophiopogon* taxa. Flowers were absent on minimondo and mondograss. Only aztecgrass (Fig. 1b) and black mondo had perigynous flowers. Variegated mondo flowers were best described as hemi-epigynous, i.e., somewhat intermediate between the two classes in morphology.

All taxa used in this study were different genetically from one another based on polymorphic molecular marker profiles (Fig. 2). Morphological approaches alone are incapable of differentiating the  $\approx \! 100$  named cultivars (Fantz, 1993), underscoring the value of a molecular marker approach for identification.

Genetic distances estimated from the molecular marker data showed little differentiation between taxa identified as *Liriope* spp. and *Ophiopogon* spp. (Fig. 2). Although trends are observable, i.e., cultivars tend to group based on genera, no significant groups existed based on AMOVA ( $P \le 0.05$ ). Most notable among the cultivars is variegated mondo. It tends to group more closely with *Liriope* spp. taxa than to *Ophiopogon* spp. taxa. Also, mor-

phological data showed variegated mondo had an intermediate floral structure between the genera. This cultivar may represent an intergeneric hybrid, if a distinction actually existed between these two genera. Cutler (1992) used various morphological characteristics to differentiate among five species of *Liriope* and 12 species of Ophiopogon. Using mostly crystal types, cuticular sculpting on cells adjacent to stomata, and leaf width, Cutler (1992) was able to assign various voucher materials to species. Yet, Cutler (1992) stated "There appear to be no anatomical reasons for regarding Liriope and Ophiopogon as separate taxa." Rudall et al. (2000) found morphological differentiation between the two genera significant, but did not find differentiation based on molecular data, substantiating, in part, the findings of our current study. Rudall (2000) also concluded that

these two genera "are clearly closely related to each other".

In conclusion, we found molecular marker data clearly differentiated the taxa from one another and showed that no duplicates existed in the samples of popular Liriopogon taxa investigated. Floral anatomy easily assigned taxa belonging to *Liriope* spp., but was less successful on *Ophiopogon* spp. taxa. Flowers were rare and ovary position less discernible on the latter. Most importantly, molecular marker data did not substantiate the existence of two genera. These preliminary data suggest that close genetic affinity exists among representative *Liriope* and *Ophiopogon* spp. taxa and support a view that these genera are possibly one.

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