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SYSTEMATICS OF THE *MECARDONIA ACUMINATA* (TRIBE GRATIOLEAE, PLANTAGINACEAE) COMPLEX OF SOUTHEASTERN USA

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By

ADJOA RICHARDSON AHEDOR
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SYSTEMATICS OF THE *MECADONIA ACUMINATA* (TRIBE GRATIOLEAE, PLANTAGINACEAE) COMPLEX OF SOUTHEASTERN USA

A DISSERTATION APPROVED FOR THE
DEPARTMENT OF BOTANY AND MICROBIOLOGY

BY

_____
Dr. Wayne J. Elisens, chair

_____
Dr. Richard Broughton

_____
Dr. Linda L. Wallace

_____
Dr. Scott Russell

_____
Dr. Jia Li

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

DISSERTATION ABSTRACT

Seed surface morphology is known to be of taxonomic significance in some plant taxa, and has been used as diagnostic features of some families, genera and species. The tribe Gratioleae (Plantaginaceae) consists of 16 genera with worldwide distribution. Similar seed surface morphologies have been observed in some members of the tribe. This study employed scanning electron microscopy (SEM) to examine detailed seed surface scultpturings of 37 species belonging to eight genera of the tribe. Sixteen seed types were identified and unique to most genera. The overall diversity of seed surface morphology observed in the tribe Gratioleae suggests extensive but taxonomically significant seed morphological variations in the tribe. Three reticulate seed types were identified for the genus *Mecardonia* that has three species endemic to the USA. *Mecardonia acuminata*, a widespread species in southeastern USA consists of at least three subspecies (*acuminata, peninsularis* and *microphylla*). Inter-simple sequence repeat markers (ISSR) were employed to elucidate the genetic variation of 23 populations in the species complex. Morphological examinations of the individuals sampled across the entire range of the species were also performed to evaluate subspecies diagnostic features and to assess the actual distributional range of each of the subspecies. Analysis of ISSR markers confirmed a widespread distribution of subspecies *acuminata* and identified populations with high genetic diversities occurring mainly in the southern ranges of the species. The ISSR analysis also revealed some populations of subspecies *microphylla* that were originally considered to be populations of subspecies acuminate. Morphological analyses also revealed possible broad historical range

distributions of subspecies *peninsularis* and *microphylla* that occurred throughout most of the range of subspecies *acuminata.* Clinal variations were also observed in some characters particularly leaf length which was found to increase from south to north across the distributional range. Regional biogeographic analysis of the morphological data revealed separation of individuals endemic to southern Florida.

CHAPTER 1

SEED MORPHOLOGY IN TRIBE GRATIOLIEAE (PLANTAGINACEAE) AND ITS TAXONOMIC SIGNIFICANCE

ABSTRACT

Seed morphology provides important taxonomic characters that can be used to infer relationships among plant genera and species. The tribe Gratioleae is made up of terrestrial to aquatic herbs with non-alveolated (non-honeycomb) endosperm. The tribe consists of 16 genera and about 182 species distributed worldwide. Scanning electron microscopy (SEM) was employed to evaluate variation in testa surface patterns of 37 species representing eight genera in the tribe Gratioleae. Fiftteen seed types were identified based on testa surface sculpturing. Seed surfaces were mostly reticulate with radial walls of high or low relief and varied tangential wall patterns. The combination of radial wall thickenings and tangential wall patterns suggests varying seed types that are unique to most genera. Similar seed types were observed among some species of the genus *Gratiola* and its segregate monotypic genus *Sophronanthe*, and the genus *Bacopa* and its segregate monotypic genus *Benjaminia*. These observations suggest that the segregation of *Sophronanthe* from *Gratiola* and *Benjaminia* from *Bacopa* are ambiguous. The seed types observed for the genus *Mecardonia* also a segregate of *Bacopa* were significantly different from those of *Bacopa*. The distinct differences in seed morphology observed for these two genera support the segregation of *Mecardonia* from *Bacopa*. Seed surfaces of the genus *Stemodia* were either striate or reticulate. Four seed types were identified for the genus *Stemodia* alone, suggesting seed morphological variations in the genus. This observation indicates that the taxonomic placement of some species of the genus is ambiguous. The overall diversity of seed surface morphology observed in the tribe Gratioleae suggests extensive but taxonomically significant seed morphological

variations in the tribe. The current study also demonstrates the taxonomic significance of intricate seed surface sculpturing and the importance of employing SEM in plant systematics.

INTRODUCTION

Seed surface morphology provides many useful taxonomic characters that can be used to distinguish genera and some species in the order Lamiales (Ichaso, 1978; Thieret 1954; Elisens, 1985). Evidence of the taxonomic significance of seed surface ornamentations has been documented in various plant families including the Scrophulariaceae (Thieret, 1954; Ichaso, 1978), Hydrangeaceae (Hufford, 1995) and Annonaceae (Svoma, 1998). Seed morphology can be used to infer relationships within families (Hufford, 1995; Svoma, 1998), within tribes (Elisens and Tomb, 1983, 1985; Takahashi, 1993), and within genera (Mathews and Levin, 1986; Ness 1989). For example, investigations of seed morphology have been used to support relationships within the tribes Antirrhineae of Plantaginaceae (Elisens and Tomb, 1983), and Pyroloideae of Ericaceae (Takahashi, 1993); and within the genera *Cordylanthus* and *Orthocarpus* of Orobanchaceae (Chuang and Heckard, 1972, 1983); *Paulownia* of Paulowniaceae (Vujicic, 1993), and *Nama* of Hydrophyllaceae (Bacon and Bragg,1986). Despite the reliability of seed morphology in taxonomy, it has been less commonly used than vegetative and floral characters, or nuclear and chloroplast markers (Barthlott, 1981, Albach & al., 2005).

Several seed morphological variations of taxonomic significance have been identified among some members of the order Lamiales particularly in the families Plantaginaceae and Orobanchaceae (Ichaso, 1978; Thieret, 1954, 1967). Light microscopic seed examinations of four genera of tribe Antirrhineae (Plantaginaceae) endemic to Brazil by Ichaso (1978) revealed five seed types unique to those genera. Subsequent Scanning Electron Microscopy (SEM) seed examinations on representative species of all sections of that tribe by Elisens and Tomb (1983)

revealed a total of seven morphological categories based on seed shape and surface ornamentation. Examinations of seed coat ornamentations in the family Orobanchaceae revealed three seed coat types in the genus *Orthocarpus* as well as many species-level differences (Chuang and Heckard, 1972, 1983). Seed surface investigations of four species of the genus *Agalinis* were found to be taxonomically significant for species identification (Canne, 1980). Similarly, seed surface ornamentations of the genus *Aureolaria* (Orobanchaceae) and genus *Angelonia* (Plantaginaceae) were also found to be taxonomically significant for species identification (Canne, 1980; Moro & al., 2001).

Previous light microscopic seed investigations of members of the tribe Gratioleae (Scrophulariaceae s.l) endemic to Central America (Thieret, 1954) revealed four main seed types including the reticulate seed type in the genera *Bacopa* and *Lindernia,* and longitudinally furrowed seed type in the genus *Stemodia*. Subsequent light microscopic investigations of 99 species of the family Scrophulariaceae (s.l.) that included tribe Gratioleae revealed 17 seed types in six tribes (Ichaso, 1978). In that study, six seed types were identified for tribe Gratioleae and at least 3 seed types for the genus *Stemodia* alone. Reticulate seed types were predominantly observed for some members of tribes Digitalideae and Buchnereae and most members of tribe Gratioleae. Thieret (1967) also observed similarities between seed characters of the genera *Bacopa* and *Scoparia* in tribe Gratioleae and Albach & al., (2005) suggested that seed characters may be useful for characterizing tribe Gratioleae.

Despite taxonomic evidence of the importance of seed surface morphology in some genera of the family Scrophulariaceae (s.l), very few detailed SEM studies have been conducted on the identification of seed types at the tribal or subtribal levels. The tribe Gratioleae as presented by Bentham and Hooker (1876) comprises 37 genera distributed worldwide particularly in North and South America, Australia and Africa. The tribe was characterized by an evoluted corolla tube, four or two stamens with two distinct anther locules, and capsules with two or four valves. One of the most important characters that distinguish the tribe is the seed morphology, which includes smooth or furrowed endosperm and longitudinal ridges with hook-like wall thickenings (reticulate) of the testa cells (Rahmanzadeh & al., 2005). Wettstein (1895) also recognized 37 genera in the tribe but excluded eight genera formerly classified by Bentham and Hooker (1987) namely, *Herpestis, Microcarpaea, Mimulus, Limnophilia, Beyrichia, Bonnaya, Vandelia* and *Sibthorpia*. Wettstein's classification of tribe Gratioleae, however included *Achetaria, Ambulia, Brythophyton, Dizygostemon, Geochorda, Ildefonsia, Lindernia, Mimetanthe, Otacanthus* and *Bacopa* and was revised to include *Herpestis* (Wettstein, 1891; Albach & al., 2005). Recent molecular phylogenetic studies using two or three plastid genes by Olmstead and Reeves, (1995) and Olmstead & al., (2001) recognized 25 genera of Wettstein's Gratioleae as part of the "Scroph II" clade, thereby excluding 12 of his genera. The tribe Gratioleae of the "New" Plantaginaceae (APG 1998; 2003; Olmstead, R. G. 2001; Oxelman & al., 2005), as proposed by Albach & al (2005), corroborated with the "Scroph II" of Olmstead and Reeves (1995).

Fischer (2004) in his recent treatment of the family Scrophulariaceae (s.l.), classified the family into 3 subfamilies; Antirrhinoideae, Gratioloideae, and Digitalidoideae. Subfamily Gratioloideae was further divided into 5 main tribes; Gratioleae, Angeloniaeae, Stemodieae, Limoselleae and Lindernieae (Fischer 2004). According to Fischer's classification, the tribe Gratioleae is made up of terrestrial to aquatic herbs with non-alveolated (non-honeycomb) endosperm. The tribe consists of 16 genera and about 182 species that can be grouped into three subtribes; Caprarinae, Dopatrinae and Gratiolinae. Subtribe Gratiolinae is characterized by herbs that are mostly aquatic, with opposite leaves, racemose or frondose inflorescence with subrotate to 2-lipped corolla and two to four stamens (Fischer, 2004). The fruit is a capsule and seeds of most members have been reported to be mainly reticulate or striate (Fischer, 2004; Ichaso, 1978; Thieret, 1954; 1967). Subtribe Gratiolinae consists of ten genera and about 121 species with temperate and tropical America or Pantropical distribution (Fischer, 2004; Pennell, 1935, 1946; Thieret, 1954). The ten genera belonging to this subtribe are *Bacopa* and *Mecardonia* (formerly *Herpestis*), *Amphianthus, Gratiola, Sophronanthe, Benjaminia, Scoparia, Boelkea, Maeviella* and *Braunblequetia* (Fischer, 2004). In a recent molecular phylogenetic analysis of the enlarged family Plantaginaceae using nuclear and plastid DNA regions (Albach & al., 2005), the genera *Bacopa, Mecardonia, Scorparia, Gratiola, Otacanthus* and *Stemodia* formed a well supported clade (Bootstrap = 90%). The first four genera of this lineage are also members of subtribe Gratiolineae, therefore partly supporting Fischer's classification of the subtribe. Similarly, a phylogenetic study of members of the order Lamiales using four plastid DNA sequences found a well supported clade

(bootstrap = 98%) for the genera *Gratiola, Scoparia, Mecardonia* and *Stemodia* (Oxelman & al., 2005). A previous molecular phylogenetic study of the family Scrophulariaceae (s.l) found a well supported clade (Bootstrap = 100%) comprising *Bacopa, Gratiola* and *Amphianthus* (Olmstead et al., 2001).

Scanning electron microscopy investigations of seed coat ornamentations provide insights into intricate microsculpturing patterns on the radial and tangential walls of the seed coat cells Canne, 1979, 1980; Chang and Heckard, 1972, 1983; Vujicic, 1993; Juan & al., 1994; Moro et al., 2001). Although previous seed surface studies of some members of Plantaginaceae by Thieret (1954; 1967) and Ichaso (1978) have suggested that reticulate seed type is common in tribe Gratioleae, particularly in subtribe Gratiolinae, and it has been observed that there is some similarity between seed characters of *Bacopa* and *Scoparia* (Thieret, 1967), no detailed SEM observations of seed surface ornamentations have been conducted at the tribal or subtribal level. The present paper presents SEM investigations of seed morphology in the subtribe Gratiolinae (Fischer, 2004). The investigations include species of eight of the ten genera classified under the subtribe as well as some species of the genus *Stemodia* (Table 1). The genus *Stemodia* was included in the current study since previous morphological studies (Bentham and Hooker, 1876; Wettstein, 1895; Small, 1913; Bigazzi, 1993) and recent molecular phylogenetic studies of the enlarged family Plantaginaceae (Olmstead & al., 2001; Albach & al., 2005; Oxelman & al., 2005) have found the genus to be closely related to some members of subtribe Gratiolinae, and the group was referred to as the ‘core” Gratioleae in Albach et al., 2005). In Fischer’s treatment of the Scrophulariaceae, the genus *Stemodia* is

classified under tribe Stemodieae (Fischer, 2004) The relevance of this investigation is therefore, to identify similarities or differences in seed surface ornamentations among genera of the subtribe Gratioliinae and the genus *Stemodia*, and to identify new and additional morphological characters that can be used in delimiting the group. Examinations of microsculpturing of the seed coats of species of the genera will serve as additional taxonomic evidence for the tribe Gratioleae as a whole.

MATERIALS AND METHODS

Mature whole seeds were removed from herbarium specimens obtained from the Missouri Botanical Garden (MO), the Botanical Research Institute of Texas (BRIT), Vanderbilt University (VDB), New York Botanical Garden (NY), and the University of Oklahoma (OKL). Samples were obtained from 41 species representing eight genera considered closely related in taxonomic treatments of subtribe Gratiolinae in tribe Gratioleae (Fischer 2004) or indicated as members of a monophyletic clade in molecular phylogenetic analyses (Albach & al., 2005; Rahmanzadeh & al., 2005; Estes and Small in press, unpublished data) (Table 1). Eighteen species were sampled for *Bacopa*, nine species for the genus *Mecardonia*, six species for *Stemodia*, three species for *Gratiola*, two species for S*coparia* and one species each for the monotypic genera *Sophronanthe*, *Benjaminia* and *Amphianthus*. Samples of species of *Bacopa* were obtained for five of six sections (Pennell, 1946): *Bacopa* Wettstein, *Bramia* (Lamarck) Wettstein, *Chaetodiscus* (Bentham) Wettstein, *Herpestis* (C. F. Gaertner), and *Mella* (Vandelli) Wettstein. Samples of section *Silvinula* (Pennell) were not included in this study.

Seeds from multiple specimens were observed initially under the compound microscope and representative seeds were selected from one herbarium sheet to represent each species. One to four seeds per species were mounted on double-sided carbon tape affixed to aluminum SEM stubs. Specimens were sputter-coated with approximately 200 Å of gold/palladium. Seeds were examined on a JEOL-880 SEM operating at 15 kV and images digitally captured using IXRF/EDS system. Images

were prepared, and plates were assembled using Adobe Photoshop version 7.0. A list of specimen collections examined is provided in the Appendix 1-1.

RESULTS

Among species examined, seeds are numerous per capsule and range in size from 0.4 mm (*Mecardonia flagellaris*) to 1.1 mm (*Amphianthus pusillus*). Seed shapes are ellipsoidal, cylindriodal or ovoid, and vary depending on seed packaging in the capsule. A cross section of *Mecardonia acuminata* seeds shows that the seed coat consists of two layers of cells. The inner layer is made up of small and rectangular cells whereas the outer layer comprises of large cells. The radial cell walls of the outermost layer the epidermis, are thickened and project into ridges to form a reticulate pattern (not shown).

Fifteen seed patterns were identified (Table 1). Thirteen reticulate and two striate seed patterns were described based on general seed surface pattern, variation in the ornamentation of the tangential walls, and the relative height and ornamentation of the radial walls. Reticulate seeds had a surface pattern characterized by a reticulum outlined by elongated radial walls against a tangential surface without grooves or lines. In contrast, striate seeds had several longitudinal grooves present on the seed surface. Tangential wall ornamentation varied in both reticulate and striate seeds, with the structural range characterized as smooth, alveolate, corrugate, nodulate, papillate, rugulate, or verrucate. Radial walls were assessed subjectively with either 'high' or 'low' relative height, whereas radial wall ornamentation patterns were described as smooth, mammilate, or nodulate.

Species of *Mecardonia* exhibited three reticulate seed patterns (1, 2, 3) that differed in the ornamentation pattern of the radial walls. Patterns 1 and 3 were unique to *Mecardonia*, although pattern 2 was found also in *Amphianthus pusillus*. Species

of *Bacopa* were characterized by five reticulate patterns (4, 5, 6, 7, 8), which differed primarily in ornamentation of the tangential walls that were either alveolate, papillate, reticulate, or verrucate. Seeds of Bacopa generally had low radial walls, except for *B. crenata*. Reticulate pattern 5 was the most common pattern in *Bacopa* (eight species) and also was observed in seeds of *Benjaminia reflexa* (= *Bacopa reflexa* (Benth.) Loefgr. & Edwall). Reticulate patterns 9 and 10 were confined to *Gratiola* and differed in height of the radial walls and tangential wall ornamentation. Reticulate 10 seeds were present also in the segregate *Sophronanthe hispida* (= *Gratiola hispida* Pollard). Whereas both species examined in the genus *Scoparia* had seeds characterized as Reticulate 11, four unique seed patterns were observed in *Stemodia*. Two reticulate patterns (12, 13) and two striate patterns (1 2) were observed among seeds of six species of *Stemodia*. Striate seeds were observed only in the genus *Stemodia*. The 15 primary seed patterns are described below.

Reticulate Seed Patterns. Reticulate seeds characterize the Gratioleae as usually defined (Thieret 1954, Dathan 1995, Fischer 2004, Olmstead et al. 2005, Rahmanzadeh et al. 2005), although *Stemodia* with both reticulate and striate seeds has been placed in the Stemoideae by Fischer (2004) and Rahmanzadeh et al. (2005).

Reticulate pattern 1 (Figs. 1A, 1B) was characterized by smooth tangential walls and high radial walls with a mammilate ornamentation. Reticulate I seeds were observed only in the genus *Mecardonia* in *M. dianthera, M. procumbens, M. vandelloides* and *M. veronicaefolia*.

Reticulate Pattern 2 (Figs. 2G, 2H) was characterized by smooth tangential and radial wall ornamentation and high radial walls. Reticulate II seeds were

confined to *Amphianthus* and three species of *Mecardonia*: *M. acuminata, M. montevidensis,* and *M. tenella*.

Reticulate Pattern 3 (Figs. 1C, 1D) had seeds with smooth tangential walls and high radial walls with nodulate ornamentation. Reticulate III seeds only were observed for *M. flagellaris.*

Reticulate Pattern 4 (Figs. 1E, 1F) was characterized by alveolate tangential walls with smooth radial walls of comparatively low relief. This seed pattern was observed only in *B. axillaris* in Section *Mella* and *B. egensis*, *B. rotundifolia, B. salzamanii* and *B. stragula* in Section *Herpestis*.

Reticulate Pattern 5 (Figs. 1G, 1H, 1K, 1L) seeds had papillate tangential walls and smooth radial walls with low relief. It was the most common seed type in *Bacopa* and characterized the seeds of eight species in two sections: *B. aquatica, B. bacopoides, B. decumbens, B. floribunda, B. gratiloides, B. lacertosa, B. laxiflora* (all of Section *Mella*), and *B. monnieri* (Section *Bramia*). Reticulate V seeds also occurred in *Benjaminia reflexa*.

Reticulate Pattern 6 (not shown) seeds were observed only in *Bacopa crenata* of Section *Mella*. The seed coat had papillate tangential walls and smooth radial walls of high relief.

Reticulate Pattern 7 (not shown) seeds also were observed only in *Bacopa* in three species of Section *Mella* (*B. auriculata, B. bracteolata, B. sessiflora*) and *B. caroliniana* of Section *Chaetodiscus*. Reticulate VII seeds had verrucate tangential walls with smooth radial walls of low relief.

Reticulate Pattern 8 (Figs. 1I, 1J) was characterized by reticulate tangential walls and low radial walls with smooth ornamentation. Reticulate VIII seeds were observed only in *B. egensis* of Section *Herpestis*.

Reticulate Pattern 9 (Figs. 2C, 2D) had seeds with verrucate tangential walls and smooth radial walls of high relief. This seed pattern was observed only in *Gratiola aurea* and *G. neglecta*.

Reticulate Pattern 10 (Figs. 2A, 2B, 2E, 2F) was characterized by corrugate tangential walls and low radial walls with a smooth ornamentation pattern. Reticulate X seeds were observed in *Gratiola pilosa* and in the segregate genus *Sophronanthe* (*S. hisida).*

Reticulate Pattern 11 (Figs. 2I, 2J, 2K, 2L) seeds had rugulate tangential walls with smooth radial walls of low relief. Seeds with this pattern were observed for the two species examined for *Scoparia*, *S. dulcis* and *S. montevidensis*.

Reticulate Pattern 12 (Figs. 3E, 3F) had seeds characterized by verrucate tangential walls and low radial walls with a nodulate ornamentation pattern. Reticulate XII seeds were observed in *Stemodia durantifolia* and *S. lanceolata*.

Reticulate Pattern 13 (Figs. 3G, 3H) seeds were similar to Reticulate XII seeds with verrucate tangential walls and nodulate radial walls, but differed from Reticulate XII by high radial walls. Seeds with this pattern only were observed in *Stemodia schottii* and *S. stricta.*

Striate Seed Patterns. Striate seeds were observed only in two species of the genus *Stemodia***.** Two patterns were observed and differed in tangential wall ornamentation and radial wall height and ornamentation pattern.

Striate Pattern 1 (Figs. 3C, 3D) seeds combined longitudinal grooves and reticulate patterns on the seed surface. The tangential walls were verrucate and the radial walls had a low relief with a smooth ornamentation pattern. Striate I seeds were observed only in *Stemodia suffructicosa.*

Striate Pattern 2 (Figs. 3A, 3B) seeds were characterized by longitudinal grooves and a surface sculpturing pattern described as nodulate. Reticulations are not apparent or similar in appearance to those described for the reticulate seed patterns. Striate II seeds were observed only in *Stemodia verticillata*.

DISCUSSION

The identification of 12 reticulate seed types for the nine genera investigated demonstrates extensive variation in reticulate seeds. Reticulate testas observed for all species of subtribe Gratiolinae also support the monophyly of the group (Thieret, 1954; Fischer, 2004; Albach & al., 2005). Most of the large genera had two or more seed types that were unique to the genus. These unique reticulate seed types provide further taxonomic evidence in support of the monophyly of each of the genera, *Scoparia Bacopa* and *Mecardonia* (Rossow, 1987; Pennell, 1946). One seed type (XI), was identified for the two species of *Scoparia* investigated. Although this genus had limited sampling, the identification of seed type XI, can be considered a representation of the seed type for the genus. Seven seed types were identified for the genus *Bacopa* which has about 60 species distributed worldwide. The seed types of *Bacopa* were all unique to the genus, but not unique to any particular section thereby indicating that, either seed characters are not useful in section characterization or the taxonomic circumscription of the sections may be ambiguous (Pennell, 1946). None of the *Bacopa* seed types was identified in the genus *Mecardonia* which is a segregate of *Bacopa* (Pennell, 1946). Despite the variation of seed types in this genus, four of the seed types were not identified in any other genus investigated. Seed type IV was however observed in the monotypic genus *Benjaminia* (Fig. 1K, L), a segregate of *Bacopa* (Bentham, 1873), section Chaetodiscus (Pennell, 1946). Seed type V (papillate tangential walls and smooth radial walls with low relief) was identified in eight of the eighteen species of *Bacopa* examined (Table 1), and was the most common seed type observed for the genus. *Benjaminia reflexa* has long been

considered congeneric with *Bacopa* but differs in its dissected leaves and slightly connate subequal clayx lobes (D'Arcy, 1979). The shared seed type V observed between *Benjaminia* and some members of *Bacopa* provides additional evidence in support of previous classification of this genus as a species of *Bacopa* (Bentham, 1873; Pennell, 1946). The three seed types I, II and III observed for the genus *Mecardonia,* also a segregate of *Bacopa* (Pennell, 1923), were not observed in any species of *Bacopa s.s.*. The tangential walls of all these seed types are smooth, whereas those of *Bacopa* are sculptured (Table 1; Fig. 1E – J). These unique seed types of *Mecardonia* support the segregation of the genus from *Bacopa* (Pennell, 1946; Rossow, 1987). Seed type II of *Mecardonia* was also observed in the genus *Amphianthus*. However, the seeds of *Amphianthus* are much bigger (1.1 mm long) - about double the size of *Mecardonia* seeds (0.4 mm – 0.6 mm). The shared seed type between *Amphianthus* and some members of *Mecardonia* suggests that these two genera may be closely related than originally thought.

Seed examination of the genus *Gratiola*, revealed 2 main seed types, IX (*G. aurea* and *G. neglecta*) and X (*G. pilosa*). Seed type X was similar to that observed for the genus *Sophronanthe* except that, the corrugate tangential wall patterns on *S. hispida* were not as closely spaced as observed in *G. pilosa*. The taxonomic placement of *G. pilosa,* was once in the genus *Sophronanthe* and then *Tragiola* (Small, 1933). The presence of similar seed types observed for the two taxa suggests close relatedness between these two taxa. Phylogenetic analysis of members of the genus *Gratiola* using *ndhF* gene sequences revealed that G. *pilosa* is sister to *S. hispida* (Estes and Small, unpublished data). Striate seed types (Thieret, 1979) were

not identified for any of the genera examined under subtribe Gratiolinae except for the genus *Stemodia* (tribe Gratioleae/Stemodieae).

The identification of both reticulate and striate seed types for the genus *Stemodia* demonstrates variations in the seed morphology for that genus and corroborates with previous seed investigations of the genus (Thieret, 1954, Ichaso, 1978, Fischer, 2004). The taxonomic placement of the genus in the tribe Gratioleae remains ambiguous (Fischer, 2004, Albach & al., 2005). Whereas most molecular and morphological studies have included *Stemodia* under the tribe Gratioleae (Albach & al., 2005; Oxelman & al., 2005; Thieret, 1978), some morphological studies have included the genus in the tribe Stemodieae (Fischer, 2004). Both Gratioleae and Stemodieae are classified under the subfamily Gratioloideae (Fischer, 2004; Rahmanzadeh & al., 2005). Previous *Stemodia* seed investigations by Thieret (1978) revealed three seed types; reticulate, striate and granulate. In the current study, two types of reticulate seeds (XII and XIII) and two types of striate seeds (Striate I and II) were identified (Fig. 3).The presence of five seed types in six of the *Stemodia* species investigated suggests variation in seed morphology and concurs with its uncertain taxonomic placement in the family. These seed observations raise questions on the infrageneric relationships, and suggest that the genus may be polyphyletic. In an attempt to determine the phylogenetic relationships among members of the newly segregated Plantaginaceae, Albach & al., (2005) employed nuclear and plastid markers to establish well supported clades within the family. One of these clades was the Gratioleae clade of which *Gratiola, Bacopa, Mecardonia, Scoparia, Stemodia* and *Otacanthus* were referred to as the 'core' Gratioleae. Previous phylogenetic studies of

the family Scrophulariaeae s.l. using three plastid genes revealed a strongly supported group of three representatives (*Bacopa, Amphianthus* and *Gratiola*) of the tribe Gratioleae (Olmstead & al., 2001). Similarly, phylogenetic analysis of the order Lamiales employing plastid DNA sequences found strong support for the Gratioleae representatives, *Mecardonia, Gratiola, Scoparia* and *Stemodia* (Oxelman & al., 2005). Molecular evidence for further support of the 'core' Gratioleae was presented by Rahmanzadeh & al. (2005) but was based on limited sampling. These phylogenetic analyses involving *Stemodia* and some members of 'core' Gratioleae, found weak support for *Stemodia* as a sister to *Gratiola* and *Otacanthus* (ITS and rps16 intron – Albach & al., 2005), sister to *Otacanthus* (*trnL-F* region – Albach & al., 2005) or sister to *Gratiola (trnL-F* and *rps*16 or *ndhF* – Oxelman & al., 2005).The combined evidence from all these phylogenetic studies indicated that the tribe Gratioleae included *Gratiola, Bacopa, Mecardonia, Scoparia, Amphianthus, Stemodia* and *Otacanthus* (Olmstead et al., 2001; Albach & al., 2005; Oxelman & al., 2005; Rahmanzadeh & al., 2005). Most of the placement of genera belonging to the 'core' Gratioleae was concordant with the morphological revision of the tribe by Fischer, (2004) except for *Stemodia* and *Otacanthus*. However, results of this seed study indicate that sampling of *Stemodia* in any Gratioleae phylogenetic or taxonomic study is critical since some members (with reticulate seeds) may be more closely related to Gratioleae than others as is evident in the diverse seed types. The phyogenetic study of Albach & al., (2005) is the only recent study with an expanded sampling of the Gratioleae. The 'core' Gratioleae included only *Bacopa, Mecardonia, Scoparia* and *Gratiola*, and the Stemodieae included *Stemodia,* and *Otacanthus*. This clade was

well supported (Bootstrap 100%) although some of the internal nodes were weakly supported. Comparing the current seed investigations with this Gratioleae clade, we observe that the seed type of *Mecardonia* I, II and III (characterized be smooth tangential walls, high relief radial walls that are either smooth, nodulate or mammilate) may be ancestral to the tribe. *Mecardonia* and *Amphianthus* are the only genera with smooth tangential walls. This infers that smooth tangential wall may be a plesiomorphic character in the group. The diversification of seed types resulted from ornamentations of the tangential walls with a retention or reduction of high radial wall relief. Evolution of the seed types may therefore have involved minimum or no ornamentation of radial walls in the Gratioleae. The smooth radial wall is a feature that was retained in most members of the group whereas, the mammilate radial walls were reduced to nodulate as seen in *M. flagellaris* and in some species of *Stemodia*, particularly in *S. durantifolia,* which shows a clear reduction of the radial wall height (Fig 3E, F). The SEM investigations reveal that the description of Gratioleae seeds as reticulate is not adequate due to variations in tangential wall patterns. The reticulate morphology of the seeds is due to the presence of smooth and distinct radial walls that appear like ridges when observed under low power magnification. The tangential wall patterns are obscure due to their intricate designs and are discernible only under high power magnification. Nevertheless, these tangential wall patterns are critical in characterizing the various genera.

Results of the investigations, suggest that seed morphology is a significant taxonomic character that can be employed in delimiting the tribe and provides evidence supporting the monophyly of the tribe (Thieret, 1979; Fischer, 2004).

Variations in the seed surface morphology are consistent for most genera and are characteristic of a genus (Thieret, 1979). The diverse seed variations observed in the 'core' Gratioleae (subtribe Gratioliinae) demonstrate the reliability of seed surface morphology in the taxonomic characterization of members of the group. These observations concur with previous findings on the taxonomic significance of seed morphology in the family Scrophulariaceae (Elisens and Tomb 1983; Chuang and Heckard, 1983, 1992; Canne, 1979, 1980; Ichaso, 1978; Moro et al., 2001). The results obtained also corroborate the current classification of *Mecardonia* as a segregate genus but raises questions on the taxonomic placement of *G. pilosa* and its relationship with the genus *Sophronanthe*.

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Table 1. Seed surface characteristics among species of tribe Gratioleae.

Genus	Section	Species	Illustration (Figure)	Seed Type	Tangential wall Ornamentation	Radial Wall Relative Height	Ornamentation
***Amphianthus* Torr.**							
		pusillus Torr.	2G, 2H	Reticulate II	smooth	high	smooth
***Bacopa* Aubl.**							
Bacopa		*aquatica* Aubl.	1G, 1H	Reticulate V	papillate	low	smooth
Mella		*auriculata* Robins.		Reticulate VII	verrucate	low	smooth
Mella		*axillaries* Benth.		Reticulate IV	alveolate	low	smooth
Mella		*bacopoides* Benth.		Reticulate V	papillate	low	smooth
Mella		*bracteolata* (Pennell) Standl.		Reticulate VII	verrucate	low	smooth
Chaetodiscus		*caroliniana* (Walter) Robinson		Reticulate VII	verrucate	low	smooth
Mella		*crenata* (Beauv.) Hepper		Reticulate VI	papillate	high	smooth
Mella		*decumbens* Fernald.		Reticulate V	papillate	low	smooth

Table 1 continued

Genus				Tangential wall	Radial Wall	
Section	Species	Illustration (Figure)	Seed Type	Ornamentation	Relative Height	Ornamentation
Herpestis	*egensis* Poeppig	1I, 1J	Reticulate VIII	reticulate	low	smooth
Mella	*floribunda* R. Brown		Reticulate V	papillate	low	smooth
Mella	*gratiloides* Chamisso		Reticulate V	papillate	low	smooth
Mella	*lacertosa* Standl.		Reticulate V	papillate	low	smooth
Mella	*laxiflora* Benth.		Reticulate V	papillate	low	smooth
Bramia	*monnieri* (Linn) Penn.		Reticulate V	papillate	low	smooth
Herpestis	*rotundifolia* Gaertn.		Reticulate IV	alveolate	low	smooth
Herpestis	*salzamanii* (Benth.) Edwall	1E, 1F	Reticulate IV	alveolate	low	smooth
Mella	*sessiflora* Benth.		Reticulate VII	verrucate	low	smooth
Herpestis	*stragula* Fern.		Reticulate IV	alveolate	low	smooth
***Benjaminia* Mart.**						
reflexa (Benth) D'Arcy		1K, 1F	Reticulate V	papillate	low	smooth

Table 1 continued

Genus / Section	Species	Illustration (Figure)	Seed Type	Tangential wall Ornamentation	Radial Wall Relative Height	Radial Wall Ornamentation
***Gratiola* Linn.**						
	aurea Pursh	2C, 2D	Reticulate IX	verrucate	high	smooth
	neglecta Torr.		Reticulate IX	verrucate	high	smooth
	pilosa Michx.	2A, 2B	Reticulate X	corrugate	low	smooth
***Mecardonia* Ruiz & Pav.**						
	acuminata (Walter) Penn.		Reticulate II	smooth	high	smooth
	dianthera (Swartz) Penn.		Reticulate I	smooth	high	mammilate
	flagellaris (Charm. & Schl.) Penn.	1C, 1D	Reticulate III	smooth	high	nodulate
	grandiflora (Benth.) Penn.		Reticulate II	smooth	high	smooth
	montevidensis (Spreng) Penn.		Reticulate II	smooth	high	smooth
	procumbens (Mill.) Small	1A, 1B	Reticulate I	smooth	high	mammilate
	tenella (Charm. & Schlecht) Penn.		Reticulate II	smooth	high	smooth

Table 1 continued

Genus Section	Species	Illustration (Figure)	Seed Type	Tangential wall Ornamentation	Radial Wall Relative Height	Radial Wall Ornamentation
	vandelloides (H.B.K.) Penn.		Reticulate I	smooth	high	mammilate
	veronicaefolia (Charm. & Schl.)		Reticulate I	smooth	high	mammilate
***Scoparia* Linn.**						
	dulcis Linn.	2I, 2J	Reticulate XI	rugulate	low	smooth
	montevidensis (Spreng) R.E. Fries	2K, 2L	Reticulate XI	rugulate	low	smooth
***Sophronanthe* Benth.**						
	hispida Benth	2E, 2F	Reticulate X	corrugate	low	smooth
***Stemodia* Linn.**						
	durantifolia (Linn.) Swartz	3E, 3F	Reticulate XII	verrucate	low	nodulate
	lanceolata Benth.		Reticulate XII	verrucate	low	nodulate
	schottii (Holz)		Reticulate XIII	verrucate	high	nodulate
	stricta Charm, & Schlecht	3G, 3H	Reticulate XIII	verrucate	high	nodulate

Table 1 continued

Genus				Tangential wall	Radial Wall	
Section	Species	Illustration (Figure)	Seed Type	Ornamentation	Relative Height	Ornamentation
	suffruticosa Kunth	3C, 3D	Striate I	verrucate	low	smooth
	verticillata (Mill.) Hassl.	3A, 3B	Striate II	nodulate	N/A	N/A

Fig 1. Reticulate seed patterns of *Mecardonia*, *Bacopa*, and *Benjaminia*. A-B, Reticulate I, smooth tangential walls and high mammalate radial walls, *Mecardonia procumbens*. C-D, Reticulate III, smooth tangential walls and high nodulate radial walls, *Mecardonia flagellaris*. E-F, Reticulate IV, alveolate tangential walls and low smooth radial walls, *Bacopa salzamanii* G-H, Reticulate V, papillate tangential walls and low smooth radial walls, *Bacopa aquatica*. I-J, Reticulate VIII, reticulate tangential walls and low smooth radial walls, *Bacopa egensis*. K-L, Reticulate V, papillate tangential walls and low smooth radial walls, *Benjaminia reflexa.* Scale: A, E, G, K = 50 um; B, C, F, H = 20 um; D, L = 10 um.

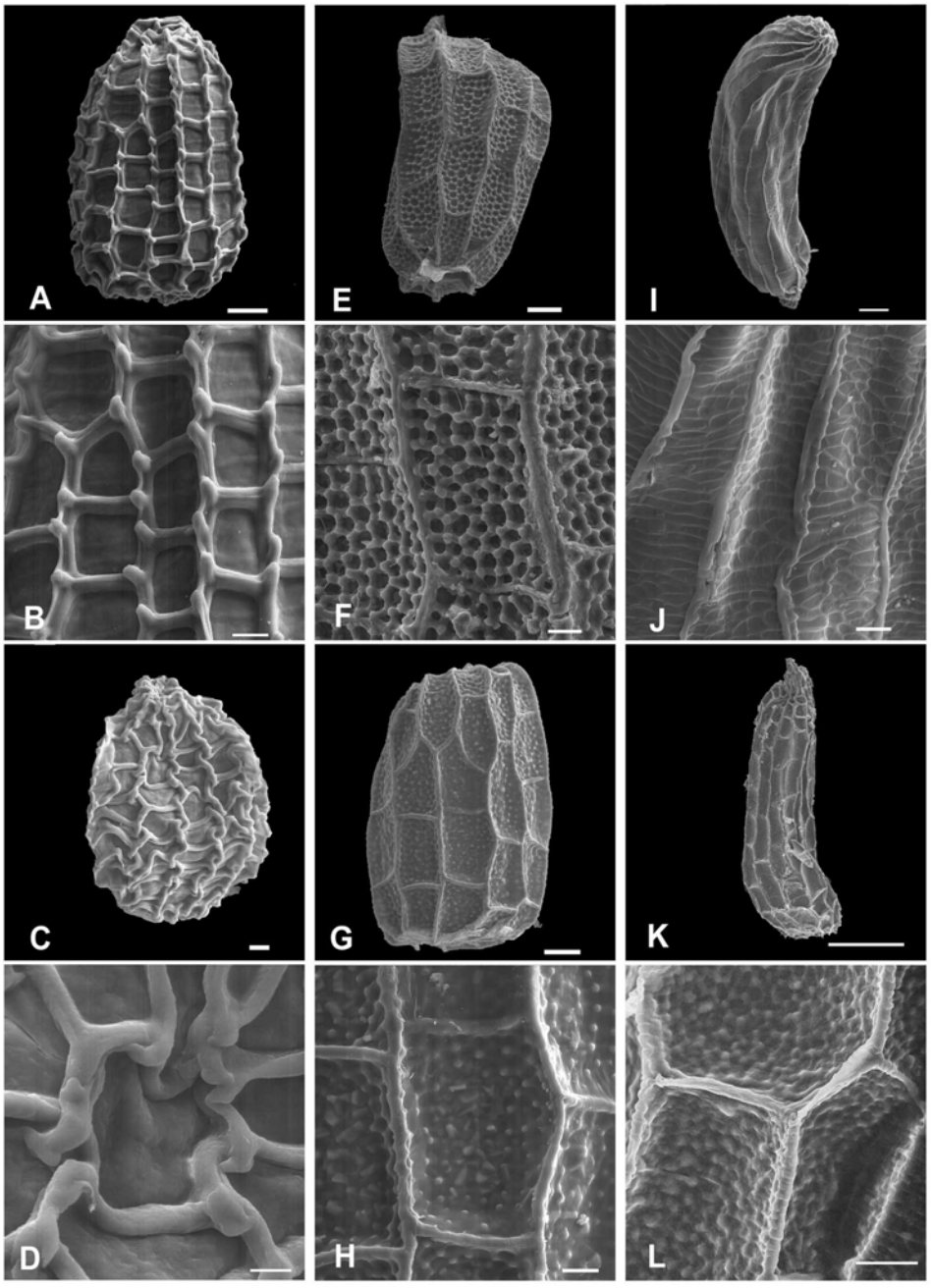
A
E
I
B
F
J
C
G
K
D
H
L

Fig 2. Reticulate seed patterns of *Gratiola*, *Sophronanthe*, *Amphianthus*, and *Scoparia*. A-B, Reticulate X, corrugate tangential walls and low smooth radial walls, *Gratiola pilosa*. C-D, Reticulate IX, verrucate tangential walls and high smooth radial walls, *Gratiola aurea*. E-F, Reticulate X, corrugate tangential walls and low smooth radial walls, *Sophronanthe hispidi*. G-H, Reticulate II, smooth tangential walls and high smooth radial walls, *Amphianthus pusillus*. I-J, Reticulate XI, regulate tangential walls and low smooth radial walls, *Scoparia dulcis*. K-L, Reticulate XI, regulate tangential walls and low smooth radial walls, *Scoparia montevidensis.* Scale: A, C, E, I, K = 50 um; B, D, F, H, J = 10 um; L = 5 um.

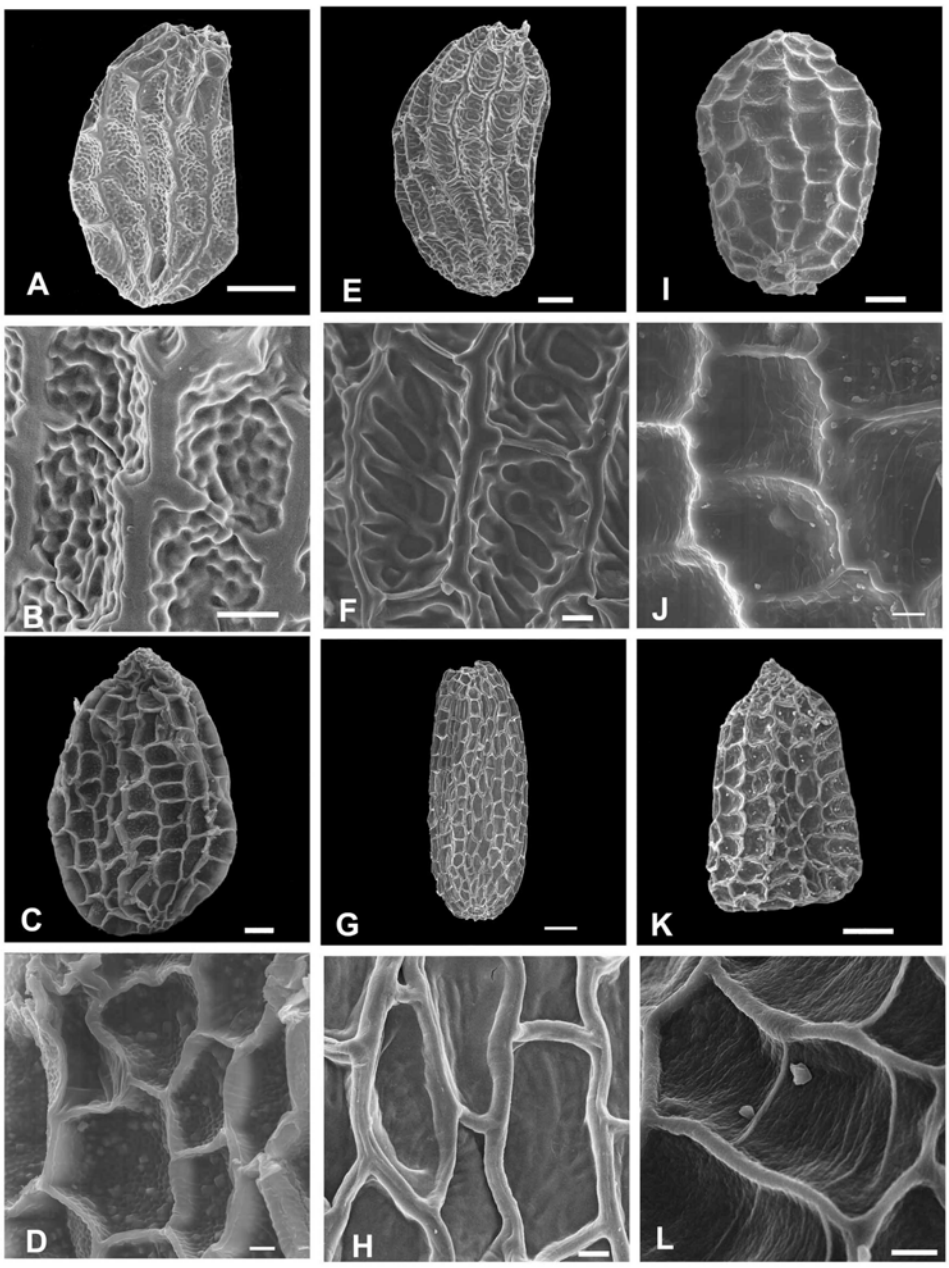
A
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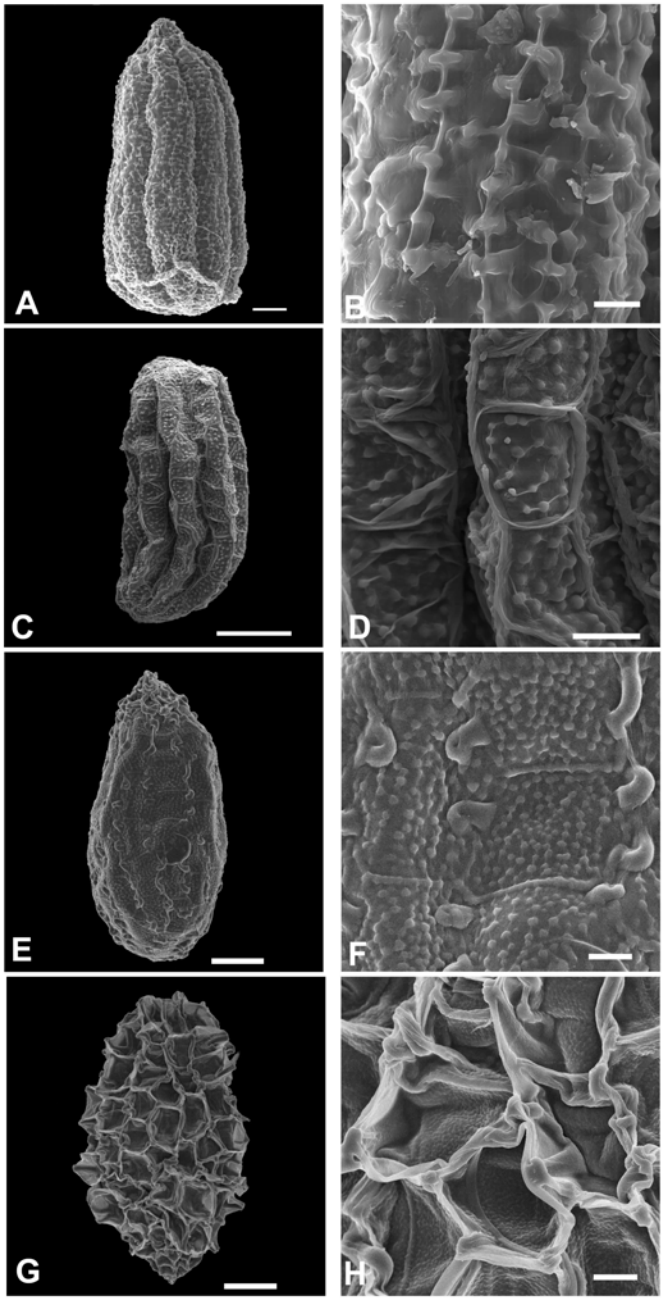
Fig: 3. Reticulate and striate seed patterns of *Stemodia*. A-B, Striate II, nodulate seed surface, *S. verticillata*. C-D, Striate I, verrucate tangential walls and low smooth radial walls, *S. suffruticosa*. E-F, Reticulate XII, verrucate tangential walls and low nodulate radial walls, *S. durantifolia*. G-H, Reticulate XIII, verrucate tangential walls and high nodulate radial walls, *S. stricta*. Scale: A, E, G = 50 um; B, F, G = 10 um; C = 100 um; D = 20 um.


A
B
C
D
E
F
G
H

Appendix 1.1 Information on sources of herbarium specimens used in the seed study

Amphianthus*: Amphianthus pusillus,* S. R. Hill 27728 (BRIT), USA, S. Carolina, Lancaster Co. ***Bacopa****: B. aquatica,* van der Werff, Gonzales 5108 (MO), Venezuela, Tachira; ***B. salzamanii,*** Kral, Boom, Stergios, Aymard 71776, Venezuela, Atures; *B. egense* K Vincent 71776 MO, Dale, Thomas, LA, Union Parish; ***Benjaminia****: B. reflexa* Mass, Koek-N, Hall, ter Welle Westra 7688 (NYBG), Guyana, Karanambo. ***Gratiola****: G. aurea* W.R Faircloth, R. Norris 4801 (MO), USA, Georgia, Brooks Co.; G. *pilosa* S. T. Orzell, E. L. Bridges 19936, (BRIT), USA, Florida, Nassau Co.; G. *neglecta,* Delzie Demaree 19159 (OKL), USA, Arkansas, Lincoln Co. ***Mecardonia****: M. procumbens,* E. Palmer 200 (MO), Mexico, Tamaulipas; M. *flagellaris,* Nerulg Berro 161 (MO), Argentina, Province Cordoba. ***Scoparia****: S. dulcis,* U. T. Waterfall, C. S. Willis 14665 (OKL), Mexico, Tamaulipas; *S. montevidensis,* R. K. Godfrey 59488 (BRIT), USA, Florida, Franklin Co., ***Sophronanthe****: S. hispida,* Delzie Demaree 23095 (BRIT), USA, Mississippi, Jackson Co., ***Stemodia****: S. suffruticosa,* Camilo Diaz, S. 2007 (BRIT), Peru, Cajamarca, San Ignacio; S. *stricta,* B. Rambo 41249 (BRIT), Brasil, Rio Grande de sul, Cai; S. *durantifolia,* R. B. Hamblett 1771 (BRIT); S. *verticillata* E. Schwindt 2290 (OKL), Argentina, Igaazu. *S. lanceolata*, A. G. Schulz 7476 (BRIT) Argentina, Colonia Benitezm;

CHAPTER 2

GENETIC VARIATION BASED ON INTER-SIMPLE SEQUENCE REPEATS (ISSR) MARKERS IN THE *MECARDONIA ACUMINATA* (PLANTAGINACEAE) COMPLEX IN SOUTHEASTERN USA

ABSTRACT

Mecardonia acuminata is a wetland species in southeastern USA, distributed throughout the region. Previous classifications proposed three subspecies *acuminata, peninsularis*, and *microphylla* based on the shape and size of leaves, and length of pedicel. Subspecies *acuminata* is widespread and occurs throughout most of the distributional range of the species, whereas subspecies *microphylla* and *peninsularis* are known to occur in sympatry with subspecies *acuminata* in the southern ranges. Inter simple sequence repeats (ISSR) were employed to elucidate the genetic relationships among 23 populations of the *M. acuminata* complex sampled from 7 states ranging from Texas and Tennessee to Florida. Ninety-four loci scored for seven ISSR primers were utilized in the genetic investigation of 237 individuals. Results of the analysis revealed appreciable levels of common loci shared among most populations with only 11 population-specific loci (private alleles). Percentage polymorphism was estimated to be 100% for total populations. Moderate to high levels of genetic variation as estimated from percentage polymorphisms were observed for populations suspected to be mixed populations of two or more subspecies. Populations of low genetic variation were indicative of the predominance of one main subspecies. Neighbor-joining analysis revealed that some populations of subspecies *peninsularis* sampled from central Florida actually consisted of mixed populations of that subspecies and subspecies *acuminata*. The current study reveals more subspecies *microphylla* populations embedded within the range of subspecies *acuminata*, and also confirms a widespread distribution of subspecies *acuminata*.

INTRODUCTION

Mecardonia acuminata (Plantaginaceae) is a widespread species in southeastern USA and can be identified by its white corolla with longitudinal purple veins on the posterior side of its throat (Pennell 1947; Rossow, 1987; Wunderlin and Hensen, 2003). This species is typically found on moist sandy loam, or heavier loam soil, subacidic or acidic, usually near streams in pineland or deciduous woodland (Pennell, 1935). It occurs along the eastern and southeastern regions of the United States, from Maryland to Texas (Pennell, 1922; Rossow, 1987). Flowering occurs mainly though the summer, followed by the formation of fruits, which occur throughout the fall (Pennell, 1946; Rossow, 1987; Wunderlin and Hensen, 2003). Previous classifications of the species have included three to four subspecies (Pennell, 1922). In his classification of the species, Pennell (1935) suggested three subspecies based on the shape and size of leaves, and length of the pedicel (subspecies *acuminata, penisularis* and *microphyla*). Subspecies *acuminata* is widespread and can be found almost in the entire distributional range of the species (Pennell 1946; Rossow, 1987); subspecies *microphylla* occurs from and northern Florida through southern Georgia to southeastern Louisiana (Pennell 1946; Rossow, 1987); and subspecies *peninsularis,* occurs only in southern Florida (Pennell 1922; Wunderlin and Hensen, 2003). In his recent taxonomic revision of the genus *Mecardonia*, Rossow (1987) confirmed the taxonomy of the three subspecies of *M. acuminata*, based on the size of leaves, length of pedicel and branching patterns of the stem. A recent guide to the vascular plants of Florida by Wunderlin and Hensen (2003) also confirmed the occurrence of all three subspecies in that state. Identification of subspecies *peninsularis* and *acuminata* in the field is relatively obvious, whereas subspecies *micophylla* is ambiguous.

Despite the widespread distribution of these species in southeastern USA, no population genetic studies have been conducted to test the morphological classification of these 3 subspecies, to assess the genetic variation among the subspecies or to determine the genetic structure of the species complex. The current study employs Inter Simple Sequence Repeats (ISSR) to assess the genetic structure of the species complex and to assess genetic variation among populations of the three subspecies. Simple sequence repeats (SSR) are short tandem nucleotide repeats of about six base pairs or less that are scattered evenly throughout the eukaryotic genome (Hamada and Kakunga, 1982). The region between any two SSRs (ISSR), can be amplified and employed as markers in plant genetic studies to facilitate cultivar identification (Charters et al., 1996), assess clonal diversity and patterns of gene flow among species and populations (Wolfe and Liston 1998). Primers designed from within the SSR motifs can be used to amplify an individual's ISSR regions via the Polymerase Chain Reaction (PCR) (Wolfe and Liston 1998). The resulting amplified fragments can be resolved on agarose or polyacrylamide gels, and the band sizes scored and analyzed (Wolff and Morgan-Richards, 1998; Wolfe and Liston 1998). The principal goals of this study were genetically to test the morphological classification of the subspecies, to determine the amount of genetic differentiation and gene flow among populations of the species complex, and to determine the level of genetic variation among the three subspecies of *M. acuminata*.

MATERIALS AND METHOD

Sampling Strategy

Several herbarium specimens of *M. acuminata* were initially observed to identify locations of the three subspecies in the southeastern USA. Twenty-three populations were sampled from seven southeastern USA states: Florida, Georgia, Alabama, Mississippi, Tennessee, Louisiana and Texas (Table 1, Fig 1). Populations were named according to counties, except for Rutherford County, TN where four populations were sampled. Populations from that county were therefore named alphabetically (Table 1). The sampling locations comprised two populations of subspecies *microphylla,* three populations of subspecies *peninsularis*, and 17 populations of subspecies *acuminata* (Fig 1). The two populations of subspecies *microphylla* were also located in the distributional range of subspecies *acuminata*. Two of the three populations of subspecies *peninsularis* also occurred in the distributional range of subspecies of subspecies *acuminata*, and the third population from Polk County, FL occurred in the distributional range of subspecies *peninsularis*. This population sampled from Polk County, FL (FL polk) was therefore considered an outgroup since it occurred outside the range of the two other subspecies. Four of the 17 southern populations of subspecies *acuminata* were also located in the range distribution of subspecies *microphylla* (GAwilc, MS georg, LA stTm, AL covi). A total of eight populations sampled from the southeast occurred in the distribution region of overlap of two or three subspecies. Eleven individuals were sampled for most populations, except for very small populations with few individuals. A total of 237 individuals were sampled. Leaf tissues were silica-dried and stored in the freezer.

ISSR Amplification

DNA was extracted from leaves using a modified CTAB method of Doyle and Doyle (1990). Fifty ISSR primers obtained from the University of British Columbia (UBC) were screened and seven primers that revealed both intra and inter-population variations were selected for the study (Table 2). For each individual, ISSR regions were amplified with a single primer at a time via PCR. Total reaction mixtures of 25uL consisted of 1.0 uL DNA, 15uM primer, 1.25 mM dNTP, 5U/uL Taq, 50 mM $MgCl_2$, 1X *Taq* polymerase buffer. The PCR was performed on a MiniCycler (MJ Research Inc., South San Francisco, CA) with the thermocycler program set at 1.5 min at 94^{o}C; 35 cycles of 40s at 94^{o}C, 45s at 45^{o}C, 1.5 min at 72^{o}C; 40s at 94^{o}C, 45s at 45^{o}C, 5 min at 72^{o}C (Wolfe and Randle, 2001). All experiments conducted included negative control reaction mixtures that had all ingredients except DNA. The PCR products were resolved on a 1.5% agarose gel in 1X TAE and a 100 bp standard marker ladder was loaded alongside to determine the size of the fragments. Gels were stained with ethidium bromide, images visualized in UV light and digitally captured with a camera and Kodak Digital Science ID software. Images were saved as Tiff files and fragment sizes determined using Kodak ID Image Analysis software. Loci for each of the primers were assigned based on fragment sizes, and ISSR data scored as diallelic, 1 (band present) and 0 (band absent).

Data Analysis – Genetic Diversity

A combined data matrix of 1s and 0s (diallelic) was generated for all populations and primers. From this matrix, levels of genetic variation were assessed

from the total number and range of loci per primer, and the number of genotypes obtained per primer. Number of genotypes per primer was estimated as the number of unique banding patterns. The total number of ISSR loci was estimated for each population. From this estimate, loci that occurred in 12 (52%) or more populations were identified as common loci. Population-specific loci (private alleles) were identified as loci or locus occurring in only one population but absent in all other populations. The ISSR data were analyzed using POPGENE version 1.31 (Yeh et al., 1999). Estimates of percentage polymorphism (P) for each population and total populations were calculated as the number of polymorphic loci divided by the total number of loci obtained for a primer Nei (1987). The mean genetic identity coefficients (I) for pairs of populations, measures of genetic similarity were computed using models of Nei (1978).

Data Analysis – Genetic Relationships

Phylogenetic relationships among populations were determined by employing the neighbor-joining algorithm. The diallelic data matrix was formatted as a NEXUS file, gaps treated as missing and the analysis conducted using PAUP 4.0 (Swofford, 2003) with distance (minimum evolution) set as the optimality criterion. Individual primer data were analyzed separately and the minimum evolution (ME) score determined for each primer. Minimum evolution is a distance method that minimizes the sum of the lengths of the branches of a phylogenetic tree (Van de Peer, 2003). The sum of the tree length (S) is estimated as:

$$S = \sum_{i=1}^{2n=3} v_i$$

where n is the number of populations in the tree (23) and v_i is the ith branch (Salami, 2003). The topology of the resulting trees from the individual primer data analyses were compared to identify sister populations or groups of closely related populations that were consistent with most data. Next, data from all primers were combined systematically starting from primer data with the lowest ME score and similar tree topologies. The relevance of this stepwise analysis was to identify reliable primers and to exclude primer data with systematic or sampling errors that may increase evolutionary "noise" caused by high levels of homoplasy. Nei's genetic distances were estimated for pairs of populations using POPGENE 1.31 and the new data formatted as a NEXUS file and analyzed using PAUP 4.0 (Swofford, 2003). The phylogram obtained was imported into Treeview (Win 32) 1.6.6 (Page, 2001). Nei's genetic distances (D) were calculated as

$D = -\text{In}\,[G_{xy}/\pi G_x G_y]$

where G_x and G_y are the frequencies of the ith allele in populatins x and y (Nei's 1978).

RESULTS

Genetic Variation

Ninety four loci were scored for all seven primers, with a range of eight (UBC 842) to 18 (UBC 836) loci per primer (Table 2). The fragment sizes varied for each primer. The largest band size range was scored for UCB 836 (180 – 2700 bp), and the smallest range scored for UCB 842 (200 – 900 bp). The number of genotypes (unique banding patterns) per population ranged from one (no variation among individuals of a population) to 11 (variation in all individuals of a population). The total number of loci per population scored for all seven primers ranged from 51 (AL lawr) to 82 (FL levy); of these, 47 - 68 were common loci found in 12 or more populations (Table 3). Private alleles were detected in only five populations (Table 3). MS georg had six loci, TN ruth B had two, FL levy, LA stTm and LA winn had one locus each (Table 3). Percentage polymorphism (P) was 100 % for total populations but moderate to high for individual populations. The populations with high P values were TN ruth B (58.95%), FL levy (57.89%), TN ruth D (56.87%) and MS geor (55.79%). These P values are above the average (50.5 %) reported for plant species (Crawford, 2003). Populations with the lowest P values were TN bedf (32.63%), FL polk (36.68%), AL lawr and AL alln (36.84%). The mean genetic identity coefficients (I) for pairwise populations ranged from 0.775 (LA alln – FL polk (FL1 – GA1)) to 0.957 (AL lawr – TN ruthB (AL1 – TNR2)) (Table 5). The low identity coefficients were observed between distant populations of different subspecies, whereas the high coefficients were observed for closely located populations that may belong to the same subspecies.

Genetic Relationships

The NJ minimum evolution scores for the individual primer data ranged from 2.051 (UBC 36) to 2.854 (UBC 9) (not shown). Tree topologies for individual primers were similar, except for UCB 812, and therefore data from the latter primer were excluded from further phylogenetic analyses. The dendrogram with a minimum evolution of 0.955 obtained from the neighbor-joining analysis suggests two main clusters of populations (Fig 2). The data matrix of pairwise genetic distances employed in the neighbor-joining analysis is shown in (Table 4). The first cluster comprises populations sampled mainly from the northwestern region of the complex, and the second cluster comprises populations sampled mainly from Florida and the Gulf Coastal Plains. The Tennessee populations were found to be polyphyletic since six of the eight populations clustered together. Populations FL polk, FL calh and TN wils were found to be distantly related to the two main clusters (Fig 2). Population FL polk is a population of subspecies *peninsularis*, whereas FL calh is a population of subspecies *microphylla*. Although genetic relatedness was observed between TN wils and FL polk, the differences in branch lengths of both pairs at the same node suggests that FL polk has undergone more genetic changes and accumulated more mutations than TN wils. This further suggests that FL polk is ancestral to TN wils. Present/absence (diallelic) character data sets such as those of ISSR have been found to be less suitable for parsimony and distance analysis compared to multistate-character data sets (Simmons et al., 2006). Comparative analysis of phylogenetic relationships of the genus *Amaranthus* (Amaranthaceae) using ITS (internal transcribed spacer), AFLP (amplified fragment length polymorphism) and ISSR

markers revealed that the ISSR neighbor-joining tree had much lower bootstrap values than the AFLP- based tree and ITS-based tree (Xu and Sun, 2001). Parsimony analysis also indicated higher retention indices (RI) in AFLP-based analysis than ISSR-based analysis.

DISCUSSION

Genetic Diversity

Increased variability of loci allows for the detection of population-specific markers that may provide important information on the genetic structure of a species (Wyttenbach et al., 1999). Markers such as ISSRs are highly variable and have revealed high genetic variability in natural populations including *Cycas* (Xiao et al., 2004), and *Botrichium* (Camacho and Liston 2001). In *Cycas*, ISSR analysis revealed low genetic diversity in endangered populations and high levels of genetic differentiation among all populations (Xiao et al., 2004) that were originally not detected with allozyme markers (Yang and Meerow, 1999). In the current study, the overall number of loci scored for the seven primers demonstrates the genetic variability of ISSR markers and its potential for identifying population-specific loci (Xiao et al., 2004). However, the presence of very few population-specific loci in the *M. acuminata* species complex suggests that the populations evolved recently, and are not completely diverged from one another although morphologically, three subspecies have been identified (Rossow, 1987). The absence of population-specific markers in 18 of the 23 populations, and the presence of high levels of common loci in all populations further supports the low level of divergence in the species complex. Alternatively, some populations may be mixed populations of two or more subspecies occurring in sympatry and possibly exchanging genes. Recent morphological studies of the species complex revealed mixed populations of two or more subspecies in several locations. The morphological studies also revealed intermediates of two or more subspecies embedded within the range of one or more subspecies.

The presence of large numbers of common loci, high total percentage of polymorphic loci (100 %) detected in all populations suggest gene flow among populations, or the effects of historical events. Historical events may include recent evolution of the species, or an initial widespread distribution of the species followed by fragmentation of populations into subpopulations during the Pleistocene. A recent morphological study of the *M. acuminata* complex indicated a fragmented broad range of subspecies *microphylla* and *peninsularis* occurring within the range of subspecies *acuminata*.

The high levels of genetic variation particularly observed in the southern populations such as FL citrus, FL levy, FL libe, MS geor, and LA stTm (total number of loci $\geq$ 68, P > 52%) all of which occur within the region of overlap, support the distribution of more than one subspecies in that area (Pennell, 1946, Rossow, 1987, Wunderlin and Hensen, 2003) although morphologically, these populations appear like single subspecies. Subspecies *microphylla* is considered to be rare and has been sited in very few southern locations including Calhoun County, FL (Wunderlin and Hensen, 2003). The lower genetic variability observed for FL calh relative to other southern populations with higher genetic variability indicates that the population may constitute one main subspecies (subspecies *microphylla*) instead of mixed populations of two or more subspecies, thereby supports the predominant occurrence of subspecies *microphylla* in that area (Wunderlin and Hensen, 2003). Low levels of genetic variability may reduce the potential of a population to survive in a changing environment (Ellstrand and Elam, 1993). The position of FL calh on the dendrogram suggests that it is distantly related to the other populations including FL libe.

Evidence from this ISSR study also confirms the sympatric distribution of subspecies *microphylla* and *acuminata* as evident in the position of FL libe on the phylogram although these two subspecies are not completely diverged from each other (Fig 2). The rarity of subspecies *microphylla* may be due to gene flow with subspecies *acuminata* that has resulted in shared morphological characteristics and genetically mixed populations of the two subspecies. For example, results of ISSR analysis of *Micromeria* (Lamiaceae) on the Canary Islands reflected patterns of introgression that has resulted in homogenization of genotypes of different species on the island (Meimberg et al., 2006).

The populations of FL citr and FL levy morphologically appear like *peninusularis* although they occur within the boundaries of subspecies *peninsularis* and *acuminata* (Wunderlin and Hensen, 2003). The lack of clustering of these populations with FL polk suggests that these two populations are not closely related to FL polk. The clustering of FL levy and FL citrus with other populations of subspecies *acuminata* indicate that these two Florida populations may be mixed populations of the two subspecies although morphologically they resemble subspecies *peninsularis*. The higher levels of genetic variation observed for these two populations compared to that observed for FL polk, suggests possible gene flow between subspecies *peninsularis* and *acuminata* and further support gene flow among subspecies. The low level of genetic variation observed in FL polk is also evidence of the occurrence of a single subspecies in that population. Similarly, lower genetic variation were estimated for most northwestern populations that occur in the range

distribution of one subspecies such as TN bedf, TN mars, TN wils, TN ruth A & B, AL lawr and AL fran and LA alln (Table 3).

Genetic Relationships

The topology of the neighbor-joining (NJ) dendrogram suggests that the subspecies are not significantly diverged from one another (Fig. 2). The NJ tree also shows that the groupings of populations are not subspecies specific. The two main clusters suggest a southern and northwestern cluster of populations. Populations TN ruth D and LA winn, exhibited high levels of loci and polymorphism indicating mixed populations of two subspecies in the northern ranges (Table 3). FL polk of subspecies peninsularis was not found to be closely related to the other Florida populations. This may be due to the absence or reduced presence of subspecies *acuminata* or subspecies *microphylla* individuals in that population. The distant relatedness of population TN wils from other Tennessee populations suggests that the population is diverging. Previous botanists have proposed the possibility of three or four subspecies of *M. acuminata* all occurring in southeastern USA (Rafinesque, 1840; Pennell, 1922).

Systematic and biogeographic implications

The current ISSR analysis reveals high genetic diversity in most southern populations. This region of distribution overlap of two or all three populations mostly constitutes the Gulf Coastal Plain that is known to harbor large numbers of endemic

genera and species (Avise, 2000; Crawford, 2003; Soltis, 2006). It is believed that the Plain may have served as a refuge for many species during the Pleistocene glaciations (Delcourt and Delcourt, 1981; Estill et al., 2001; Crawford, 2003). The flora in the Gulf Coastal Plain is considered to be of geologically-recent origin (Thorne, 1993). Southeast of this Plain is the restricted dominant distribution of subspecies *peninsularis*. The climate in this region is subtropical and may account for the restricted range of the subspecies.

The basal position of the southern populations particularly FLPolk in the dendrogram suggests that this population maybe ancestral to the other populations. The higher genetic diversity observed in FLlevy and FL citrus indicates they may be mixed and ancestral populations or may represent the center of diversity of the species from which two or more subspecies evolved. Subspecies *microphylla* maybe a recent subspecies based on its rarity, low genetic variation and position of FL calh on the dendrogram. However, distance trees depict mathematical optimizations of genetic distances and greatly reduce phylogenetic information (Van de Peer, 2003), thus the evolutionary history of populations cannot be assumed from the topography of the dendrogram. The lack of grouping of some of the Tennessee populations is consistent with recent reports of several phylogeographic breaks in that area (Soltis et al., 2006). The high genetic diversity observed for TN ruthB and TN ruth D both from Rutherford County in Tennessee, concur with results of the morphological studies that revealed a likely historical occurrence of subspecies *peninsularis* in that area.

The absence of a significant level of private alleles in the species complex and the presence of common loci further suggests that the species complex represents a

single evolutionary lineage with three or more incompletely differentiated morphological subgroups, or represents remnants of a once widespread subspecies *peninsularis* and *microphylla* occurring in mixed populations with subspecies *acuminata*. Gene flow among individuals of the three subspecies occurring in sympatry may be resulting in homogenization of genotypes in the species complex.

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Table 1. Locations of 23 populations sampled from *M. acuminata* complex in southeastern USA

Population (County/State)	Code	Sample Size	Location Latitude (^{o}N)	Longitude (^{o}W)
Subspecies *peninsularis*				
Florida - Citrus	FL citr	11	28.7295	82.2715
Florida - Levy	FL levy	11	29.4415	82.6365
Florida - Polk	FL polk	11	28.3109	82.0561
Subspecies *microphylla*				
Florida - Liberty	FL libe	11	30.2043	84.7483
Florida - Calhoun	FL calh	11	30.4072	85.1622
Subspecies *acuminata*				
Alabama - Covington	AL covi	11	31.1718	86.2908
Alabama - Franklin	AL fran	11	34.4820	87.6490
Alabama - Lawrence	AL lawr	11	34.4880	87.5007
Georgia - Wilcox	GA wilc	11	31.9488	83.5589

Table 1 continued.

Louisiana - Allen	LA alln	11	30.5185	93.0152
Louisiana - Beauregard	LA beau	10	30.5100	93.2328
Louisiana - St. Tammany	LA stTm	11	30.4962	90.1988
Louisiana - Winn	LA winn	11	35.7532	92.9170
Mississippi - George	MS geor	11	30.7791	88.7171
Tennessee - Bedford	TN bedf	11	36.6772	86.5223
Tennessee - Marshall	TN marsh	7	35.6251	86.8105
Tennessee - Maury	TN maur	11	35.5872	86.8975
Tennessee - Rutherford	TN ruth A	11	35.7394	86.5955
Tennessee - Rutherford B	TN ruth B	7	36.6551	86.4576
Tennessee - Rutherford C	TN ruth C	11	35.8738	86.2844
Tennessee - Rutherford D	TN ruth D	11	36.0590	86.4847
Tennessee -Wilson	TN wils	11	36.0274	86.3673
Texas - Nacadoches	TX nacd	11	31.6190	94.6832

Table 2. Attributes of ISSR primers used to generate markers from 237 individuals sampled from the *M. acuminata* complex.

Primer	Sequence	Total No. of Loci (= 94)	Range of Fragment Sizes (bp)	No. of Genotypes Per Population
UBC 807	$(AG)_8T$	14	215 – 1400	4 - 11
UBC 809	$(AG)_8G$	13	204 – 1500	2 - 9
UBC 812	$(GA)_8A$	15	220 – 1400	2 - 11
UBC 815	$(CT)_8G$	14	230 – 1500	3 - 11
UBC 836	$(AG)_8YA$	18	180 – 2700	3 – 11
UBC 842	$(GA)_8YG$	8	200 – 900	1 - 7
UBC 845	$(CT)_8RG$	12	260 – 1700	3 – 10

Table 3. Genetic variability at 94 ISSR loci in 23 of *M. acuminata* species complex. P = Percentage of polymorphic loci. Common loci are present in 0.53 % of populations sampled; Private alleles (loci) are observed only in one population.

Population (County/State)	Total Number of loci	Number of Common Loci	Number of Population Specific Loci (Private alleles)	Percentage Polymorphic loci
AL covi	63	53	0	44.21
AL fran	57	52	0	44.21
AL lawr	51	47	0	36.84
FL calh	52	49	0	48.42
FL citrus	68	60	0	52.63
FL levy	82	68	1	57.89
FL libe	62	55	0	53.68
FL polk	55	48	0	36.68
GA wilc	61	55	0	52.63
LA alln	64	54	0	36.84
LA beau	53	51	0	51.58

Table 3 continued.

LA st.Tm	68	56	1	48.42
LA winn	70	56	1	50.53
MS geor	70	57	6	55.79
TN bedf	53	50	0	32.63
TN marsh	55	51	0	44.32
TN maury	61	55	0	47.37
TN ruth A	53	48	0	41.05
TN ruth B	65	59	2	58.95
TN ruth C	56	48	0	42.21
TN ruth D	70	62	0	56.85
TN wils	59	51	0	43.16
TX nacd	55	50	0	47.37
Total	94	-	11	100

Table 4. Nei and Li's (1978) genetic distances between pairs of populations among three subspecies in the *M. acuminata* complex.

	FL calh	LA beau	TN wils	AL lawr	TN maur	LA winn	TN ruth A	TN bedf	AL fran
FL calh	0	0.064	0.051	0.070	0.075	0.063	0.063	0.10	0.088
LA beau		0	0.076	0.051	0.106	0.107	0.069	0.149	0.068
TN wils			0	0.073	0.105	0.072	0.103	0.087	0.085
AL lawr				0	0.116	0.094	0.049	0.148	0.046
TN maur					0	0.116	0.102	0.131	0.119
LA winn						0	0.109	0.107	0.138
TN rutA							0	0.149	0.079
TN bedf								0	0.138
AL fran									0

	GA wilc	TN mars	TX nacd	TN ruth C	TN ruth B	AL covi	FL libe	TN ruthD
FL calh	0.103	0.062	0.182	0.101	0.045	0.089	0.091	0.094
LA beau	0.083	0.093	0.060	0.087	0.062	0.120	0.086	0.078

Table 4 continued

	GA wilc	TN mars	TX nacd	TN ruth C	TN ruth B	AL covi	FL libe	TN ruthD
TN wils	0.085	0.100	0.084	0.100	0.106	0.101	0.057	0.046
AL lawr	0.061	0.097	0.059	0.053	0.055	0.116	0.080	0.062
TN maur	0.083	0.086	0.113	0.129	0.110	0.126	0.102	0.101
LA win	0.084	0.143	0.137	0.103	0.147	0.090	0.077	0.079
TN ruth A	0.060	0.069	0.067	0.059	0.051	0.135	0.066	0.068
TN bedf	0.132	0.170	0.150	0.171	0.188	0.107	0.138	0.101
AL fran	0.067	0.870	0.058	0.084	0.076	0.131	0.134	0.065
GA wilc	0	0.709	0.058	0.055	0.073	0.089	0.50	0.046
TN mars		0	0.067	0.109	0.092	0.131	0.092	0.070
TX nacd			0	0.072	0.055	0.124	0.064	0.067
TN ruth C				0	0.056	0.130	0.060	0.061
TN ruth B					0	0.142	0.054	0.070

Table 4 continued

	GA wilc	TN mars	TX nacd	TN ruth C	TN ruth B	AL covi	FL libe	TN ruthD
AL covi						0	0.084	0.067
FL libe							0	0.041
TN ruth D								0

	FL levy	FL citr	LA stTm	MS geor	LA alln	FL polk
FL calh	0.083	0.073	0.050	0.040	0.095	0.073
FL beau	0.012	0.097	0.145	0.089	0.184	0.138
TN wils	0.076	0.070	0.113	0.069	0.155	0.093
AL lawr	0.083	0.083	0.142	0.097	0.226	0.136
TN maur	0.099	0.089	0.143	0.132	0.146	0.161
LA win	0.078	0.085	0.101	0.093	0.186	0.153
TN ruth A	0.116	0.105	0.132	0.062	0.227	0.152

Table 4 continued

	FL levy	FL citr	LA stTm	MS geor	LA alln	FL polk
TN bedf	0.147	0.107	0.100	0.123	0.159	0.137
AL fran	0.145	0.098	0.152	0.119	0.177	0.131
GA wilc	0.097	0.052	0.129	0.082	0.143	0.157
TN mars	0.137	0.097	0.156	0.121	0.163	0.175
TX nacd	0.123	0.094	0.141	0.090	0.137	0.153
TN ruth C	0.100	0.095	0.150	0.092	0.187	0.155
TN ruth B	0.098	0.111	0.148	0.085	0.209	0.163
AL covi	0.116	0.100	0.103	0.087	0.153	0.129
FL libe	0.063	0.074	0.120	0.044	0.123	0.140
TN ruth D	0.076	0.051	0.094	0.062	0.148	0.107
FL levy	0	0.082	0.112	0.091	0.182	0.160

Table 4 continued

	FL citr	LA st.Tm	MS geor	LA alln	FL polk
FL citr	0	0.125	0.092	0.121	0.163
LA stTm		0	0.122	0.136	0.149
MS geor			0	0.182	0.140
LA alln				0	0.256
FL polk					0

Table 5. Measures of Genetic Identity Coefficients between pairs of 23 populations sampled from the *M. acuminata* species complex. Code: ALl = ALLaw, AL2 = ALFran, AL3 = ALCovi, FL1 = FLCalh, FL2 = FLLibe, FL3 = FLLevy, FL4 = FLCitr, FL5 = FLPolk, GA1 = GAWilc, LA1 = LABeau, LA2 = LAWin, LA3 = LASTt, TN1 = TNWils, TN2 = TNMaur, TN3 = TNBedf, TN4 = TNMars, TNR1 = TNRutA, TNR2 = TNRutB, TNR3 = TNRutC, TNR4 = TNRutD, TX1 = TXNacd, LA4 = LAAlln, FL5 = FLPolk

	FL1	LA1	TN1	AL1	TN2	LA2	TNR1	TN3	AL2	GA1	TN4	TX1	TNR3	TNR2
FL1	****	0.939	0.950	0.934	0.928	0.939	0.939	0.902	0.916	0.956	0.915	0.913	0.911	0.930
LA1		****	0.927	0.950	0.900	0.900	0.933	0.861	0.935	0.921	0.911	0.942	0.917	0.951
TN1			****	0.930	0.900	0.930	0.902	0.917	0.918	0.918	0.905	0.920	0.905	0.908
AL1				****	0.890	0.910	0.953	0.862	0.955	0.940	0.908	0.942	0.948	0.957
TN2					****	0.891	0.903	0.878	0.888	0.921	0.917	0.894	0.880	0.906
LA2						****	0.897	0.899	0.871	0.919	0.867	0.872	0.902	0.874
TNR1							****	0.861	0.924	0.941	0.934	0.935	0.943	0.962
TN3								****	0.871	0.876	0.844	0.860	0.843	0.837
AL2									****	0.936	0.917	0.943	0.919	0.936

Table 5 continued.

	FL1	LA1	TN1	AL1	TN2	LA2	TNR1	TN3	AL2	GA1	TN4	TX1	TNR3	TNR2
GA1										****	0.932	0.944	0.947	0.940
TN4											****	0.936	0.897	0.923
TX1												****	0.939	0.956
TNR3													****	0.946
TNR2														****

	AL3	FL2	TNR4	FL3	FL4	LA2	MS1	LA3	FL5
FL1	0.942	0.960	0.970	0.910	0.930	0.902	0.940	0.833	0.904
LA1	0.895	0.927	0.935	0.890	0.907	0.865	0.915	0.832	0.871
TN1	0.912	0.953	0.963	0.927	0.932	0.893	0.934	0.856	0.912
AL1	0.899	0.933	0.950	0.920	0.921	0.867	0.908	0.797	0.873
TN2	0.890	0.913	0.914	0.906	0.915	0.866	0.877	0.864	0.852

Table 5 continued.

	AL3	FL2	TNR4	FL3	FL4	LA2	MS1	LA3	FL5
LA2	0.922	0.935	0.933	0.925	0.918	0.904	0.912	0.830	0.858
TNR1	0.884	0.947	0.946	0.901	0.900	0.877	0.940	0.797	0.859
TN3	0.906	0.879	0.911	0.871	0.899	0.905	0.884	0.853	0.872
AL2	0.884	0.909	0.945	0.891	0.907	0.859	0.888	0.838	0.878
GA1	0.924	0.961	0.964	0.918	0.949	0.879	0.921	0.867	0.855
TN4	0.886	0.922	0.943	0.883	0.917	0.856	0.887	0.850	0.839
TX1	0.891	0.947	0.944	0.902	0.911	0.869	0.914	0.872	0.858
TNR3	0.879	0.942	0.941	0.905	0.909	0.861	0.912	0.829	0.857
TNR2	0.868	0.947	0.933	0.907	0.895	0.863	0.918	0.812	0.850
AL3	****	0.919	0.936	0.890	0.905	0.902	0.916	0.859	0.880
FL2		****	0.960	0.939	0.929	0.887	0.957	0.882	0.870
TNR4			****	0.927	0.950	0.910	0.940	0.863	0.898
FL3				****	0.922	0.894	0.913	0.833	0.852

Table 5 continued.

	AL3	FL2	TNR4	FL3	FL4	LA2	MS1	LA3	FL5
FL4					****	0.882	0.912	0.886	0.850
LA3						****	0.885	0.790	0.862
MS1							****	0.834	0.869
LA4								****	0.775
FL5									****

Figure 1. Map of eastern USA showing locations of sampled populations in southeastern USA. Circles denote subspecies *acuminata* populations, squares denote subspecies *peninsularis* populations, diamonds denote subspecies *microphylla* populations

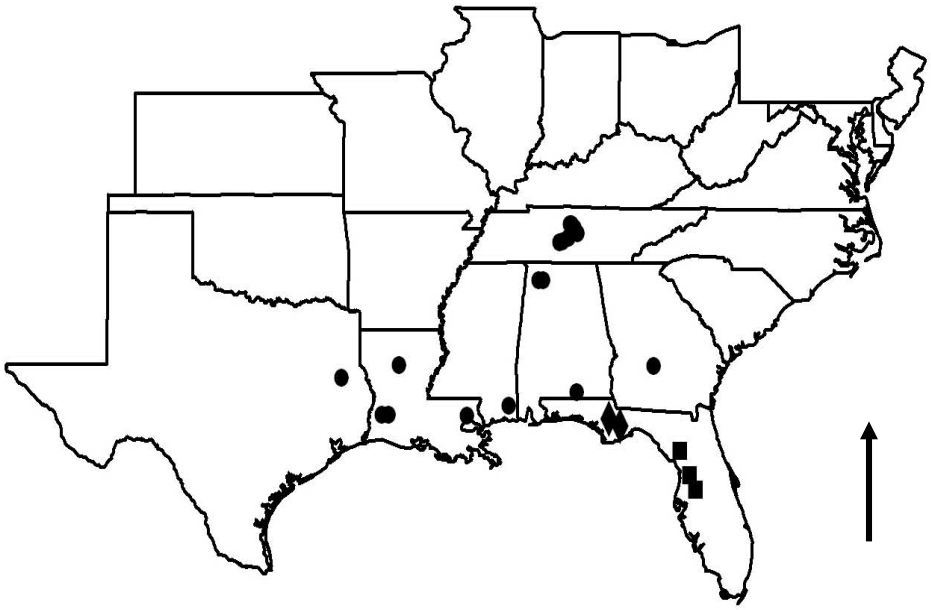
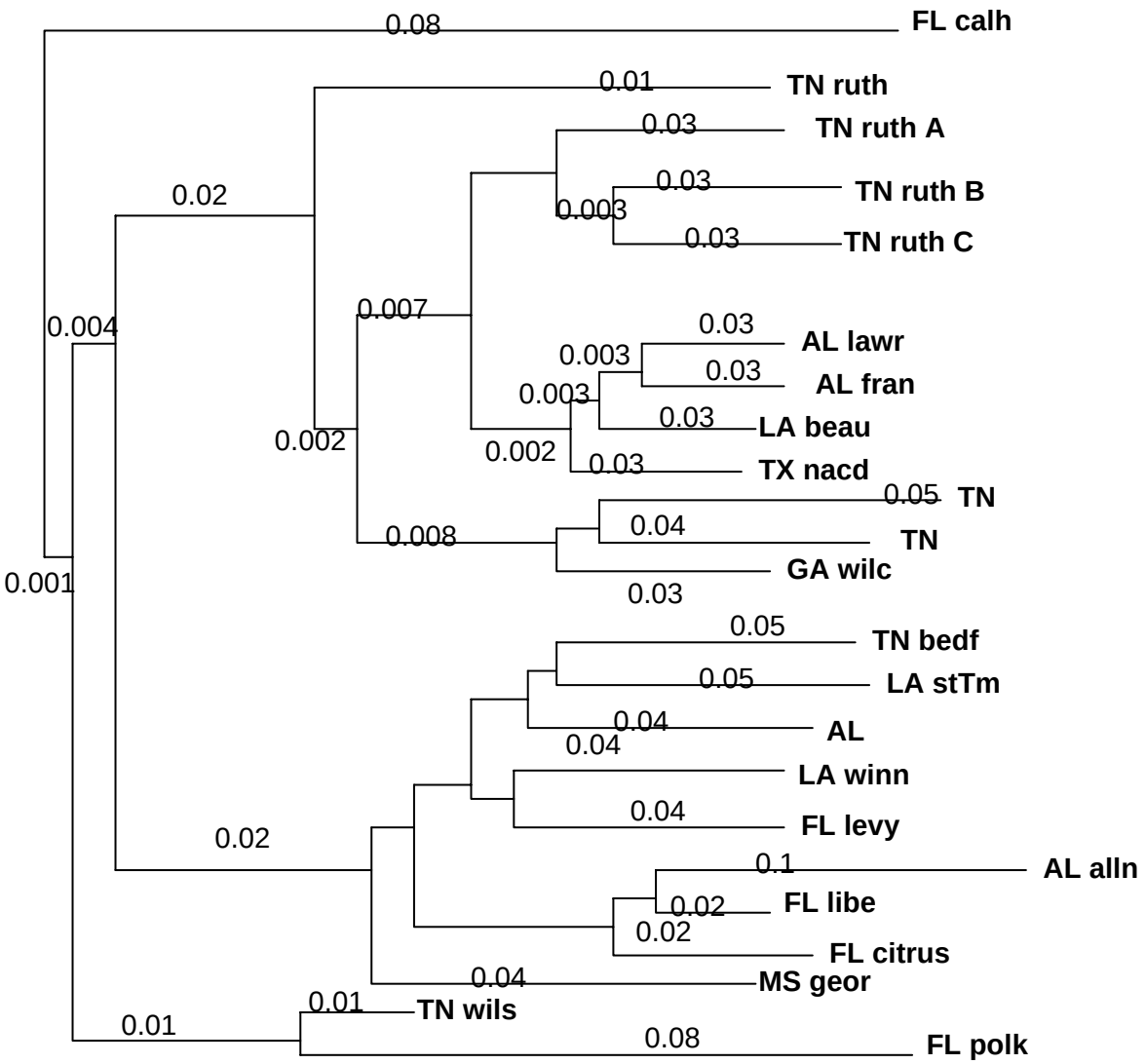


Fig 2. Neighbor-joining dendrogram showing genetic relationships among 23 populations sampled from the *M. acuminata* species complex in southeastern USA. Numbers denote branch lengths.


0.08
FL calh
0.01
TN ruth
0.03
TN ruth A
0.03
TN ruth B
0.02
0.003
0.03
TN ruth C
0.007
0.004
0.03
AL lawr
0.003
0.03
AL fran
0.003
0.03
LA beau
0.002
0.002
0.03
TX nacd
0.05
TN
0.04
TN
0.008
GA wilc
0.001
0.03
0.05
TN bedf
0.05
LA stTm
0.04
AL
0.04
LA winn
0.04
FL levy
0.02
0.1
AL alln
0.02
FL libe
0.02
FL citrus
0.04
MS geor
0.01
TN wils
0.01
0.08
FL polk

CHAPTER 3

MORPHOLOGICAL VARIATION IN THE *MECARDONIA ACUMINATA* (PLANTAGINACEAE) COMPLEX IN SOUTHEASTERN USA

ABSTRACT

Mecardonia acuminata is a classic example of a widespread endemic species in the southeastern USA. Morphological variations in the complex resulted in the classification of at least three varieties or subspecies for the species by previous botanists. However, the distributions and diagnostic features of two of the subspecies; *peninsularis* and *microphylla* are unclear due to the shared morphological features of these two subspecies and the widespread subspecies *acuminata*. The present study involved examination of three vegetative and five reproductive characters that were known to serve as diagnostic features of one or more taxa of the species. The study employed biostatistical analyses to assess character correlations and clinal variations in the complex. Leaf length was observed to increase from south to north across the distributional range and therefore, not a reliable diagnostic character north of the peninsular Florida. Discriminant function analyses were conducted to distinguish taxa based on latitude, longitude, and biogeographic associations. Two clusters of specimens were identified for all three associations. Latitudinal and biogeographic analyses both indicated separation of individuals of southern Florida origin from the remaining northern, eastern and western individuals. Longitudinal analysis indicated partial separation of eastern individuals sampled along the Atlantic Coastal region. Attempts to distinguish taxa based on branching pattern and overall subspecies characteristics revealed that branching pattern is not reliable diagnostic character. Results of this study indicate that subspecies *peninsularis* can be distinguished by its ascending peduncle angle of suspension, diffuse basal branching pattern of the shoot and small leaves especially in the southern ranges of the complex. Subspecies *microphylla* can be distinguished based on its short (< 20 mm) fruit peduncles and divaricate peduncle angle of suspension. Subspecies *acuminata* comprises individuals with divaricate peduncle angle of

suspension and long fruit peduncles (> 20mm). Subspecies *acuminata* was also observed to comprise many individuals that were intermediates of two or more subspecies. Wilks' lambda estimates confirmed similarities among taxa for some characters. These findings suggest a much broader historical range distribution of subspecies *peninuslaris* and *microphylla* than originally established. The historical populations of these two subspecies are currently integrating into subspecies *acuminata* as evident in the identification of a third branching pattern in the complex. Taxa were not identified as distinct groups in specific regions which raises questions about the subspecific classification of the species.

INTRODUCTION

Mecardonia acuminata (Walt.) Small is a perennial wetland herb or subshrub that exhibits pronounced morphological variation throughout its range from Maryland to south Florida to east Texas and southeastern Missouri. The species is one of three within the genus *Mecardonia* Ruiz & Pav. native to North America; ***M. acuminata*** and ***M. procumbens*** (Mill.) Small are congeners native to the southeastern USA (Rossow 1987). The seven remaining species of *Mecardonia* are native to South America. *Mecardonia acuminata* (axilflower) is morphologically distinct within *Mecardonia* based on its erect habit, white corollas with purple nerves, and distinctly pubescent corollas (Rossow 1987).

Morphological variation within the species has resulted in three or four infraspecific taxa proposed by Rafinesque (1840), Pennell (1935) and Rossow (1987). Three varieties or subspecies are most commonly recognized based on variation in nine morphological characters: branching pattern, leaf length, leaf shape, leaf base, peduncle length, peduncle angle in fruit, sepal length, sepal width, and corolla length. Morphological variation is most pronounced in peninsular Florida and the eastern Gulf region where all three subspecies occur with sympatric or parapatric ranges (Fig 1).

Rafinesque (1840) first recognized variation among populations of ***M. acuminata*** by naming four varieties that implicated variation in leaf shape throughout the range of the species: var. *obovata* Raf. from North Carolina, var. *microphylla* Raf. from Florida, var. *cuneata* Raf. from Carolina and Alabama, and var. *angustifolia* Raf. from Florida. Pennell (1922) carefully evaluated diagnoses of these varieties and searched in vain for Rafinesque's types, because he had described (Pennell 1920) ***M. acuminata*** var. *brevifolia* Pennell, which became synonymous with Rafinesque's (1840) earlier named var. *microphylla*. Three varieties were proposed by Pennell (1935) to accommodate morphological variation in *M. acuminata*: var. *acuminata* (=

var. *typica*), the larger-leafed variant that occurs throughout the range of the species except for southern Florida; var. *microphylla* (Raf.) Pennell, which occurs along the eastern Gulf coast and is characterized by an ovate leaf shape, cuneate leaf base, and shorter peduncle length; and var. *peninsularis* (Pennell) Pennell, which was segregated based on its shorter leaf , sepal and corolla lengths, diffusely spreading branching pattern, and distribution in southern Florida. Citing Pennell's (1935) treatment, most North American taxonomists have recognized three varieties of *M. acuminata*, which is presented widely in floristic treatments (e.g., Small 1933, Long and Lakela 1971, Wunderlin 1982, Wunderlin and Hansen 2003).

Rossow (1987) revised the genus *Mecardonia*, recognized 10 species distributed in North and South America, and presented the most thorough taxonomic and morphological study of the genus to date. He commented on the distinctness of *M. acuminata* within the genus and recognized three taxa within *M. acuminata*, but elevated them to subspecific rank: subsp. *acuminata*, subsp. *microphylla* (Raf.) Rossow, and subsp. *peninsularis* (Pennell) Rossow. Delimitation and characterization of the subspecies essentially followed Pennell's (1935) earlier treatment and differentiated subspecies based on two new characters, peduncle angle and sepal width, and four characters used by Pennell (1935): branching pattern, leaf length, leaf base, and peduncle length. We follow Rossow's (1987) taxonomy in the present study.

Summarizing variation within *M. acuminata*, subsp. *acuminata* is found throughout the geographic range of the species and is characterized by leaves longer than 25 mm, peduncles ranging between 25 – 35 mm long, and lateral branches arising at the midpoint from the stem base. Subspecies *peninsularis* occurs only in central to southern Florida, but overlaps with subsp. *acuminata* in north central Florida and is distinguished from the other subspecies by leaves less than 25 mm long and diffuse basal branching. Subspecies *microphylla* is rarely

identified and annotated in collections. It occurs from northern Florida and southern Georgia to southeastern Louisiana, and is characterized by peduncle lengths less than 12 mm and sepal widths ≥ 2 mm. Subspecies *microphylla* is sympatric with subsp. *acuminata* and plants have been identified to each subspecies within the same population (Ahedor and Elisens, unpublished data). No chromosome number determinations for *M. acuminata* are published, but the chromosome base number for *Mecardonia* is $x = 11$ based on multiple chromosome counts of $2n = 22$ for *M. procumbens* (e.g., Kaul 1974, Sinha 1988, Trivedi 1984) and one count of $2n = 44$ (Kaul 1975).

The present study was initiated to assess morphological variation throughout the range of the species and to test for character correlation among characters, geographic locations (latitude and longitude), and biogeographic regions (Soltis et al. 2006). Specimen sampling was concentrated in Florida and the Gulf Coastal Plain region where taxonomic and morphological variation is most pronounced. An additional goal was to test taxonomic hypotheses delimiting three subspecies in *M. acuminata*.

MATERIALS AND METHODS

A total of 402 specimens were examined for morphological variation from six herbaria (330 specimens) and from the authors' field collections (72 specimens) deposited at OKL.

Herbarium specimens were examined from the Botanical Research Institute of Texas (BRIT), University of Florida (FLAS), University of Georgia (GA), Missouri Botanical Garden (MO), Vanderbilt University (VDB), and the University of Oklahoma (OKL). Herbarium specimens represented individuals in 13 states throughout the geographic range of *M. acuminata*. Specimens representing collections made by the authors from 23 field populations were obtained from seven states: Florida (5), Georgia (1), Alabama (3), Mississippi (1), Tennessee (8), Louisiana (4) and Texas (1). Sources of all specimens used in the study are compiled in Appendix 3-1.

Three vegetative and five reproductive characters were evaluated; branching pattern, leaf length, leaf shape, peduncle length in flower, peduncle length in fruit, peduncle angle of suspension, sepal width and fruit length (Table 1). Three characters were qualitative (branching pattern, leaf shape, peduncle angle of suspension) and variation was assessed using multiple states (Table 2). Some reproductive characters (peduncle length in flower, peduncle length in fruit, sepal width and fruit length) were not measurable on all specimens, because of developmental differences among plants at the time of collection. Branching pattern was not recorded for all herbarium specimens since the shoot base had been removed from some specimens. Leaf length and leaf shape were recorded for all specimens. Leaf length was measured from the tip of the leaf to the base of the lamina for leaves located in the mid section of the plant (mature leaves). Floral and fruit peduncle lengths were measured from the base of the sepals enclosing the petals or fruit to the point of attachment of the peduncle to the stem. Specimens with only flowers or fruits were evaluated for characters present only. Sepal width was measured at the broadest mid-portion of the outermost sepal. Fruit length was measured from the tip of the fruit to the point of attachment of the fruit to the peduncle. Branching pattern

was assessed to be either dendriform at mid region (1), dendriform at the base (2), or intermediate (3 - dendriform at mid region but also with reduced basal branching). Peduncle angle was determined to be divaricate (1) or ascending (2). Leaf shape for each plant was determined to be ovate (1), elliptic (2), lanceolate (3), linear (4) or oblanceolate (5) (Pennell, 1935).

Morphological data were analyzed statistically using Statistical Package for Social Sciences (SPSS) ver. 15.0 for windows (SPSS, Inc. 2006), and graphs plotted using SigmaPlot ver. 10.0 (SYSTAT, 2006) and Excel (Microsoft Corp., 2003). Quantitative data were analyzed using analysis of variance (ANOVA) and Fisher's F statistics (F-values = Group mean squares/error mean square) and significance levels estimated. Calculated F-values depict the level of variability within a group of samples - the larger the F-value, the greater the variability of the sample data. Box plots were constructed to depict patterns of variation in characters (Pereira et al., 2007). Descriptive statistics were performed for qualitative characters; percentages of variables for each character were determined, and Chi square analysis was conducted to assess the level of variation in each character. Chi square is a statistical measure of how far a sample distribution deviates from a theoretical distribution χ^2 = (observed – expected) ^{2} / expected (Zar 1996).

Spearman's Rank-Order correlation analysis was performed on all data to determine relationship among pairs of characters. Correlations were tested for significance with two-tailed tests. Regression analyses were performed to determine clinal variations for each character (Henderson, 2005). Regression coefficients were calculated for each character, with longitude and latitude as the independent variables. The curves corresponding to the highest regression coefficient of five relationships (linear, logarithmic, exponential, power and quadratic) were

chosen (Schmalzel et al., 2004). Regression lines and coefficients were calculated using the Least-Squares method in Excel (Microsoft Corp., 2003).

Multiple analysis of variance (MANOVA) and discriminant function (DF) analysis were conducted (Zar, 1996; Woods et al., 2005). F-values with levels of significance and Wilks' lambda values (U-statistics), were estimated from these analyses. Wilks' lambda is a direct measure of the proportion of variance in the dependent variables (characters) that is unaccounted for by the independent variable (latitude, longitude, biogeographic regions and subspecies). Wilks' lambda is an inverse measure whereby, values near 0 denote high discrimination between groups (Everitt, B. S., and Dunn, G. 1991). Samples with missing values were excluded in each analysis. Discriminant fuction analysis was conducted to investigate differences between groups (independent variables) and to determine the most parsimonious way of distinguishing groups. Individual specimens served as operational taxonomic units (OTUs), and missing data were excluded from the analyses. Since most of the herbarium specimens were not identified to the subspecies level, DF analysis were performed to determine if samples grouped into clusters without a priori assumption of the three subspecies (Boonkerd et al., 2002; Woods et al., 2005). In these evaluations, separate DF analyses were conducted based on latitude, longitude and the five phylogeographic regions that were predetermined based on physiogeographic barriers in southeastern USA (Soltis et al., 2006). To assess the distinctness of taxa in these DF analyses, scatter plots were visually inspected for partial or distinct clusters.

The data matrix was then partitioned into subspecies based on known morphological characteristics (Pennell 1922, 1935; Rossow, 1987; Wunderlin and Hensen, 2003). Thus, *a priori* decision of subspecies was imposed in these subsequent analyses. Univariate analyses of

variance (ANOVA) were performed on quantitative characters and Chi-Square analyses were performed on qualitative characters to determine if a statistically significant difference existed among subspecies. In order to establish similarities among subspecies, Duncan's Multiple Range Test was conducted to identify subsets of subspecies for each quantitative character examined. Regression analyses were conducted to assess clinal variations in each subspecies, and DF analyses were performed to evaluate subspecies delimitations.

Diffuse branching at the base (dendriform at base) is believed to be one of the major diagnostic features of subspecies *peninsularis* (Pennell 1922, 1935; Rossow, 1987; Wunderlin and Hensen, 2003). In order to test the taxonomic significance of branching patterns, the original data matrix was again partitioned into the three observable branching patterns; dendriform at mid-point, dendriform at base and intermediate. Discriminant function analysis was conducted to determine if the different subspecies would segregate into distinct or partial clusters.

RESULTS

Morphological variation within *M. acuminata*

All specimens had been collected from Latitude 25^{o} – 38^{o} North, and Longitiude 76^{o} – 96^{o} West. Leaf lengths ranged from 7.00 mm to 45 mm long with a mean of 23.50 mm. Fruit lengths ranged from 4 mm to 9 mm with a mean of 6.50 mm. Sepal widths ranged from 1.00 mm to 2.50 mm, with a mean of 1.60 mm (Table 1). Floral peduncle lengths ranged from 5 mm to 32 mm with a mean of 17.01 mm, whereas fruit peduncles ranged from 11 mm to 38 mm with a mean of 22.70 mm (Table 1; Fig 2). Leaf shapes were mainly oblanceolate (55.3%), elliptic (41.7%), linear (2.5%), ovate (0.2%) or lanceolate (0.2%). Branching patterns of specimens were observed to be 57.6% dendriform from mid-point, 23.7 % dendriform from base, and 15.6% intermediate (Table 2). Intermediate branching pattern has not been reported in previous studies, but was observed to be prevalent in some populations especially in Arkansas, Tennessee and northern Alabama. Peduncle angle was divaricated in 65.1 % of the specimens and ascending in 32.2% of the specimens. All the characters were found to be statistically significant across latitude ($P < 0.01$) and/or longitude ($P < 0.05$) except for leaf shape (Table 4). Estimates of Fisher's F statistics (F-value) suggest that leaf length ($F = 108.95$; $P = 0$) and fruit peduncle length ($F = 42.34$; 0) (Table 1) were the two most variable quantitative characters in the species, whereas leaf shape ($\chi^2 = 479.1$; $P = 0$) and habit ($\chi^2 = 131.14$; $P = 0$) were the two most variable qualitative characters (Table 2).

Character correlations within *M. acuminata*

All characters were correlated with one or more other characters. Eleven of the 28 pairwise character correlations were significantly correlated at $P = 0$, $P \leq 0.01$ or $P \leq 0.05$

(Table 3). The strength of significant associations ranged from |r| = -0.24 (leaf length and peduncle angle) to 0.66 (floral peduncle and fruit peduncle). Leaf length and peduncle angle exhibited significant associations with both latitude and longitude (Fig. 3). Fruit length and sepal width were also correlated with latitude, whereas floral and fruit peduncles were correlated with longitude. Moderate to weak clinal variations were therefore observed for most characters from south to north (latitude) and from east to west (longitude). Leaf length exhibited the strongest clinal variation along latitude ($R^2 = 0.282$; P = 0) and longitude ($R^2 = 0.1$; P = 0) (Fig 3). These analyses indicate low but statistically-significant associations between leaf length and both latitude and longitude.

Character Correlation to Latitude, Longitude and Biogeographic Region

Multivariate ANOVA (MANOVA) for each character along latitude indicates statistically significant results ($P \leq 0.01$) for seven out of the eight characters measured (Table 4). The results indicate similarities within most characters except for leaf length and peduncle angle. No significant difference was observed for leaf shape along latitude (Wilks' Lambda = 0.944; P = 0.43). Similarities within characters were observed across longitude for habit, leaf length, peduncle angle and sepal width (Table 4). The two DF analyses on latitude and longitude did not reveal distinct but partial clusters. The first DF analysis based on latitude revealed partial separation of samples occurring along latitude 26^{o} - 28^{o} North in southern Florida, where most subspecies *peninsularis* occurs (Fig 4). Samples of latitude 29^{o} North (northern Florida Peninsular) were intermediates between subspecies *peninsularis* and the remaining *M. acuminata* cluster, but closer to the latter group. This indicates that plants in latitude 29^{o} north are morphologically similar to subspecies *acuminata* even though they have

basal branches and small leaves similar to subspecies *peninsularis*. The second DF analyses based on longitude did not reveal any significant clusters, although samples from longitude 76^{o} – 82^{o} west along the Eastern Coastal Plains were slightly separated from samples from the remaining locations (Fig 4). Both latitude and longitude DF analyses therefore support the occurrence of subspecies *peninsularis* in southern Florida but suggest a much smaller range of distribution than previously reported (Pennell 1922, 1935; Rossow, 1987; Wunderlin and Hensen, 2003). The DF analyses did not reveal a partial segregation of samples occurring along latitude 30^{o} - 31^{o} North or longitude 83^{o} – 90^{o} West where subspecies *microphylla* is known to occur. This suggests that subspecies *microphylla* is morphologically similar to subspecies *acuminata* and is embedded in the range of the latter thus, confirms the sympatric distribution of the two subspecies.

Results of DF analysis to test the effect of biogeographic barriers on the distribution of the species, showed a consistent and marked partial separation similar to that obtained for latitude (Fig 5). Region 1 (southern Florida) was partially separated from the remaining four regions (2 - 5), that did not show any apparent biogeographic pattern or separation. The lack of a biogeographic pattern for regions 2 – 5, is consistent for species exhibiting high levels of outbreeding and physiological tolerance to fluctuating environmental conditions (Fritsch and Lucas, 2000).

Character Correlation to Subspecies delimitation

Discriminant function analysis to test subspecies delimitation based on previously reported diagnostic features (Pennell 1922, 1935; Rossow, 1987; Wunderlin and Hensen,

2003), clearly separated subspecies into 3 distinct clusters (Fig 6). Seven out of the eight characters analyzed showed significant variations within each subspecies (Table 1 and 2). Fruit length was less variable in subspecies *acuminata* and *peninsularis* (Fig 2). Subspecies *acuminata* demonstrated the most variants for all characters with most outliers within the range of the other two subspecies. Discriminant function analyses conducted to test the reliability of branching patterns in distinguishing subspecies, did not resolve the data into 3 subgroups, but suggested that specimens with intermediate branching patterns can be considered intermediates of those with branching at mid point and branching at the base (Appendix 3-2). Diffuse branching at the base (dendriform at base), a character used in identifying subspecies *peninsularis* was found to be present among 18.2 % of subspecies *acuminata* and 26.7% of subspecies *microphylla* mostly occurring north of central Florida (Table 2; Fig 7). The remaining six characters measured were less variable in each subspecies, therefore reliable for delimiting subspecies. Duncan's multiple range test (Table 1) separated leaf length into 2 subsets and indicated a statistically-significant difference between subspecies *peninsularis* and the other two subspecies ($P = 0.05$). Two subsets were also obtained for both floral and fruit peduncle lengths. In each of these two characters, subspecies *microphylla* was separated from the other two subspecies. Fruit length/size of subspecies *peninsularis* was smallest with a mean of 6.0 mm, and that of subspecies *acuminata* was large with a mean of 6.56 mm. The lack of statistically-significant subsets for some characters indicates overlap of ranges among subspecies (Fig 2). Linear regression analyses for each subspecies suggest moderate clinal variations for most characters of subspecies *microphylla* (Appendix 3-3). Clinal variations in leaf length and peduncle lengths were

also observed for subspecies *peninsularis* (Appendix 3-4), but no significant clinal variation was observed for any character of subspecies *acuminata* (Appendix 3-5).

DISCUSSION

The current investigations confirm the widespread distribution of subspecies *acuminata* even at the lower latitudes (26^{o} North) where subspecies *peninsularis* is predominant. Subspecies *acuminata* exhibits a wide range of variation in all characters examined, with no significant clinal variations of characters. As latitude increases, leaf length (size) for subspecies *peninsularis* and *microphylla* increases. This latitudinal association with leaf length has also been observed in some endemic southeastern USA taxa such as *Halesia carolina* (Styracaceae) complex (Fritsch and Lucas, 2000). The leaf length latitudinal association in *M. acuminata* complex indicates that when subspecies *peninsularis* and *microphylla* occur north of their known ranges, they are not distinguishable due to their larger leaf sizes except for the short peduncle lengths in subspecies *microphylla* (Table 1; Fig 2). Larger leaves are therefore not unique taxonomic characters of subspecies *acuminata* and *microphylla* alone, but present in subspecies *peninsularis* occurring at higher latitudes. Although subspecies *peninsularis* was not readily identified in the northern ranges of the species complex, its diffuse basal branching pattern and ascending peduncle angle were observed in some members of subspecies *acuminata*. These diagnostic features found in subspecies *acuminata* suggest that subspecies *peninsularis* once had a broader distribution range than presumed by previous botanists. Subspecies *acuminata* individuals with one or two diagnostic features of subspecies *peninsularis* may be intermediates of the two subspecies. These individuals may also depict remnants of the historically broad range *peninsularis*, which are currently isolated and possibly integrating into subspecies *acuminata* populations. Evidence from

this study revealed that subspecies *peninsularis* has a sympatric distribution with other subspecies north of Florida. It was identified in the states of Georgia, Tennessee, Maryland and Texas (Fig 7; Appendix 3-6). In northern Georgia, it was found in the same county (Catoosa) as subspecies *microphylla* (Appendix 3-6). The southern populations of subspecies *peninsularis* represent relicts that were restricted to a glacial refuge in southern Florida (Pennell, 1935). It has been reported that southern Florida served as a refuge for many plants and animals during the Pleistocene (Soltis, 2006). The small leaf lengths/sizes of these populations may be due to climatic or ecological factors and not taxonomy. These climatic or ecological effects are also evident in its prolonged flowering season (Pennell, 1935). Most members of subspecies *acuminata* and *microphylla* sampled from southern Florida had small leaf lengths ranging from 8 mm to 20 mm.

The separation of specimens from southern Florida (most of Florida Peninsular) from the rest of the specimens by the DF analyses confirms the climatic, biogeographic or ecological impact of that region on the morphology of the species. This biogeographic pattern has been observed in a few angiosperms in SE USA including *Liriodendron tulipifera* (Sewal et al., 1996; Parks et al., 1994). Lack of a biogeographic pattern on the distribution of subspecies *acuminata* and *microphylla* north of Florida Peninsula, indicates effective dispersal mechanism(s) of the species irrespective of physiogeographic barriers. No clear geographic patterns have been found in some plant species occurring in the region including *Liquidambar styraciflua* (Soltis et al., 2006), *Prunus* (Shaw and Small, 2005) and *Arabidopsis thaliana* (Jorgensen and Mauricio, 2004). Although the dispersal mechanisms of ***M. acuminata*** complex were not examined in this study, pollen is known to be dispersed by bees. The small seed sizes of the species

(< 0.5 mm) may be easily dispersed by wind and water (my personal observation). Physiogeographic barriers in southeastern USA such as, rivers and high elevations therefore may not pose barriers to the species dispersal. Species distributed over one or more climatic belts spanning latitudes, often possess clines for physiological characteristics and their associated vegetative characters (Stebbins, 1950). These clines result from adaptive responses to the environmental conditions prevailing in the different parts of the species range (Fritsch and Lucas, 2000; Spurr and Barnes, 1980). Thus, the partial separation of subspecies *peninsularis* occurring in southern Florida may be due to subtropical climatic effects on leaf size in that region and other physiological characteristics that were not evaluated in this study. Clustering of specimens from latitude 29^{o} North in central Florida with other northern specimens (north of Latitude 29^{o} North) of subspecies *microphylla* and *acuminata* instead of specimens from southern Florida (Figure 4), suggest these assumed subspecies *peninsularis* specimens are probably intermediates of subspecies *peninsularis* and one or two other subspecies particularly, subspecies *acuminata*. This region may be a major contact or suture zone of subspecies *peninsularis* and one or two subspecies, where individuals may have hybridized and exhibit diffuse basal branches similar to subspecies *peninsularis*. It has been documented that extensive hybridization of species and genera may have occurred in the Florida Peninsular refuge during the Pleistocene when many taxa were forced into close proximity (Edwards et al., 2006; Soltis et al., 2006).

It is apparent from the current investigations that the major diagnostic characters of subspecies *microphylla* are shorter floral and fruit peduncles. This confirms earlier reports by Pennell (1935) and Rossow (1987). Although short floral peduncles were

observed in some members of subspecies *acuminata*, their corresponding fruit peduncles were relatively long (> 20 mm). Due to the many shared characteristics of the two subspecies and their sympatric distribution, identification of subspecies *microphylla* is still problematic especially prior to its reproductive stage. The current investigations also revealed that subspecies *microphylla* is fragmented but widespread in the range distribution of the complex particularly, in the western ranges (Fig. 7). Eastern Texas and western Louisiana, where most of these subspecies *microphylla* populations were detected was also once a Pleistocene refugium (Swenson and Howard, 2005). The observed distribution of subspecies *microphylla* in the southeastern USA suggests a historical discontinuous distribution separated by the Mississippi river. This pattern is similar to that observed for loblolly pine, *Pinus taeda* (Al-Rabab'ah and Williams, 2002; Soltis et al., 2006). Few samples of subspecies *microphylla* were also observed in southern Florida (Charlotte County) where subspecies *peninsularis* is prevalent (Appendix 3-6). Therefore, subspecies *microphylla* may not be as rare as originally thought but, may either be distributed within the range of the other two subspecies as low-density populations, or was originally widespread and continuous but now fragmented. Paleoecological reconstructions of postglacial tree distributions suggests that many temperate species were restricted to the southern latitudes during the last ice age, but rapidly dispersed northwards following glacial warming (Davis, 1981; Delcourt and Delcourt, 1987). On the other hand, Bennett (1985) had suggested that temperate species of North America may have occurred in low densities much across the continent even during most of the glacial periods. It was therefore inferred from the current investigations that subspecies *microphylla* can be found in at least 10 states as oppose to

five as earlier reported (Pennell, 1935; Rossow, 1987). The sympatric distribution of this subspecies also suggests a fairly recent evolution possibly from subspecies *acuminata*. However, these hypotheses cannot be confirmed from the current study. The taxonomic circumscription of subspecies *microphylla* is further supported by results of DF analyses which demonstrate that when all eight characters examined are combined, three distinct three subspecies are identified including subspecies *microphylla* (Fig. 6).

Taxonomic Implications

The moderate clinal variations observed in *M. acuminata* complex raises questions about whether the clines developed from primary differentiation or secondary contact. The vegetative (leaf length) and reproductive (floral and fruit peduncle lengths) clinal variations, and overlap of characters such as habit, fruit length and sepal width suggest secondary contact of previously differentiated entities that are currently mixing freely. The current widespread distribution of the species in southeastern USA may be due to a northward range expansion following the Pleistocene and Quartenary glaciation events in eastern USA. The presence of subspecies *peninsularis* diagnostic characters in other subspecies, the sympatric distribution of subspecies *microphylla* and the identification of a third habit in the complex may be evidence of a historically broader range distribution of all three subspecies that have undergone fragmentation but currently exhibiting ongoing evolutionary processes. Results of this study depict complex evolutionary processes in *M. acuminata* that are masked by distinct but inconsistent morphological features. In view of these evidences, the taxonomic classification of the

three taxa forming the *M. acuminata* complex as subspecies is ambiguous. Classification of the taxa as varieties as originally classified by Pennell (1935) is more justified since taxa were not identified as distinct entities occurring in specific regions but occurred throughout the distributional range and exhibit clinal variations in most of the diagnostic features.

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Table 1. Variation among quantitative morphological characters in the *Mecardonia acuminata* complex. (N = samples size, S.D. = standard deviation, F = Fischer's statistics). Letters a and b denote subsets of taxa based on characters as obtained from Duncan's Multiple Range Test. *** = $P \leq 0.001$, ** = $P \leq 0.01$, * = $P \leq 0.05$

Quantitative Character	Taxon	N	Mean (S.D)	F (ANOVA)
Leaf Length (mm)	*M. acuminata*	406	23.50 (7.05)	108.95***
	subsp *acuminata*	346	25.11b (5.77)	4.20***
	subsp *peninsularis*	48	11.29a (2.13)	1.74
	subsp *microphylla*	16	22.53b (8.02)	2.69
Floral Peduncle Length (mm)	*M. acuminata*	298	17.01 (5.40)	13.23***
	subsp *acuminata*	251	17.20b (5.22)	2.58**
	subsp *peninsularis*	40	17.13b (5.89)	1.16
	subsp *microphylla*	10	9.0a (3.62)	1.49

Table 1 continued.

Quantitative Character	Taxon	N	Mean (S.D)	F (ANOVA)
	M. acuminata	338	22.73 (4.94)	42.34***
Fruiting Peduncle Length (mm)	subsp *acuminata*	285	23.17b (5.21)	2.66**
	subsp *peninsularis*	41	22.46b (6.12)	1.88
	subsp *microphylla*	16	12.00a (1.50)	4.64*
	M. acuminata	331	6.50 (0.84)	7.56***
Fruit Length (mm)	subsp *acuminata*	280	6.56b (0.87)	0.83
	subsp *peninsularis*	39	6.0a (0.80)	0.88
	subsp *microphylla*	15	6.13ab (0.83)	0.63

Table 1 continued.

Quantitative Character	Taxon	N	Mean (S.D)	F (ANOVA)
	M. acuminata	387	1.60 (0.36)	7.16***
Sepal Width (mm)	subsp *acuminata*	328	1.62a (0.35)	2.03**
	subsp *peninsularis*	47	1.42a (0.32)	2.50**
	subsp *microphylla*	15	1.61a (0.49)	0.59

Table 2. Variation among qualitative morphological characters in the *M. acuminata* complex (N = sample size).

	Taxon	N	PERCENT OBSERVED			Chi-Square value (χ^2)
			Basal	Mid-Point	Intermediate	
	M. acuminata	395	23.7	57.6	15.6	121.4***
Dendriform	subsp *acuminata*	340	18.2	69.2	18.8	131.14***
Branching	subsp *peninsularis*	45	71.1	28.9	0	8.02*
	subsp *microphylla*	15	26.7	73.3	0	3.27
			Divaricate		Ascending	
	M. acuminata	405	61.5		32.2	47.184***
Peduncle	subsp *acuminata*	339	72.9		28.0	65.49***
Angle	subsp *peninsularis*	48	25		75	12.00***
	subsp *microphylla*	15	80		20	5.4*

Table 2 continued.

	Taxon	N	PERCENT OBSERVED					Chi-Square
			Ovate	Elliptic	Lanceolate	Linear	Oblanceolate	value (χ^2)
	M. acuminata	405	0.2	41.7	0.2	0.2	55.3	556.171***
	subsp *acuminata*	345	0.3	43.2	2.3	2.3	55.9	479.1***
Leaf Shape	subsp *peninsularis*	48	0	33.3	0	0	66.7	5.33*
	subsp *microphylla*	15	0	40.0	0	13.3	46.7	2.8

Table 3. Correlation among morphological characters to latitude and longitude in the *M. acuminata* complex based on Pearson's Rank-Order Correlation Analysis. Significance values: * 0.05, ** 0.01, *** 0.001

	Habit	Leaf Length	Leaf Shape	Peduncle Angle	Floral Pedun. Length	Fruit peduncle	Fruit Length	Sepal Width	Lat.	Long.
Habit	1.0	-0.087	0.122*	0.154**	-0.062	-0.03	0.065	0.05	-0.001	-0.09
Leaf Length		1.0	-0.046	-0.24**	-0.01	0.099	0.333**	0.248**	0.531***	0.307**
Leaf Shape			1.0	0.001	0.076	0.022	-0.154**	-0.002	0.044	0.037
Ped. Angle				1.0	116*	0.069	-0.075	-0.013	-0.219**	-0.342**
Floral Ped. L					1.0	0.660***	-0.041	-0.132*	-0.049	-0.148*
Fruit Peduncle						1.0	0.153**	0.018	0.003	-0.186**
Fruit Length							1.0	0.259**	0.151**	0.006
Sepal Width								1.0	0.196**	0.064
Latitude									1.0	0.249**
Longitude										1.0

Table 4. Results of multivariate analyses (MANOVA) of *M. acuminata* complex showing extent to which morphological characters differ across latitude/longitude, and among biogeographic regions and subspecies. Significance values: * 0.05, ** 0.01, *** 0.001; df1 (degrees of freedom for number of groups per analyses); Lat. = 13, Long, = 20, Biogeography = 4, Subspecies = 2; df2 (degree of freedom for total number of samples evaluated) = 208

Statistic	df1	Branching Form	Leaf Length	Leaf Shape	Peduncle Angle	Floral Peduncle Length	Fruit Peduncle	Fruit Length	Sepal Width
Wilks' Lambda									
LAT	12	0.875	0.636	0.944	0.741	0.876	0.887	0.888	0.865
LONG	20	0.830	0.705	0.913	0.788	0.901	0.889	0.885	0.842
BIOGEOG	4	0.905	0.716	0.983	0.873	0.952	0.948	0.984	0.953
SUBSPECIES	2	0.975	0.668	0.998	0.871	0.903	0.790	0.946	0.974
F-Value									
LAT		2.483**	9.919***	1.021	6.051***	2.454**	2.215**	2.185**	2.705**
LONG		2.052**	4.185***	0.947	2.697***	1.101	1.215	1.302	1.876**
BIOGEOG		5.671***	21.467***	0.941	7.824***	2.714*	2.953*	0.873	2.687**
SUBSPECIES		2.709	53.265***	0.230	15.832***	11.431***	28.494***	6.129**	2.81

Fig. 1. Map showing distribution range of *M. acuminata* in southeastern USA (shaded area) and the five biogeographic regions in southeastern USA.

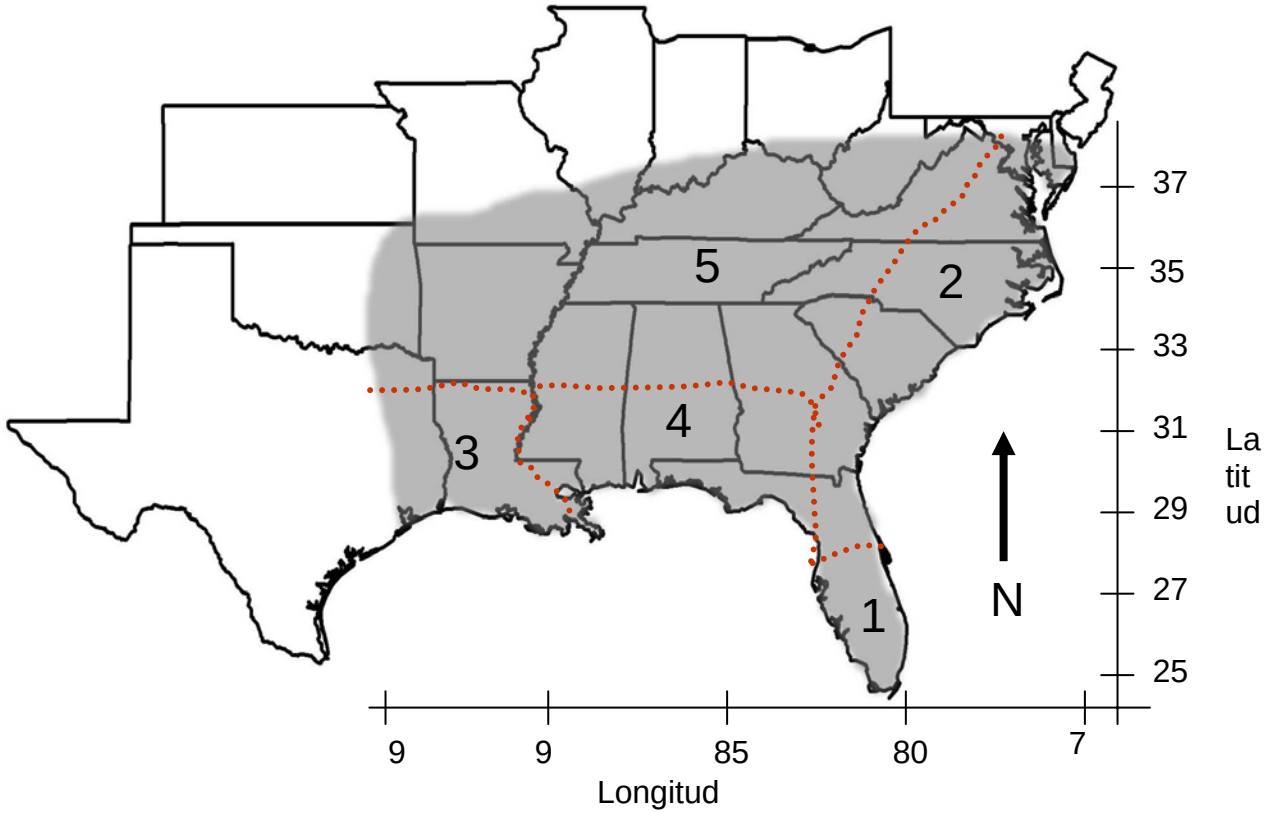
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Figure 2. Box Plots illustrating variation in five morphological characters in three subspecies of the *M. acuminata* complex. Means denoted by vertical bars (|), shaded boxes indicate 50% of variation ranging from 25th to 75th percentile. Horizontal bars delimit 10th and 90th percentile; dots denote outliers.

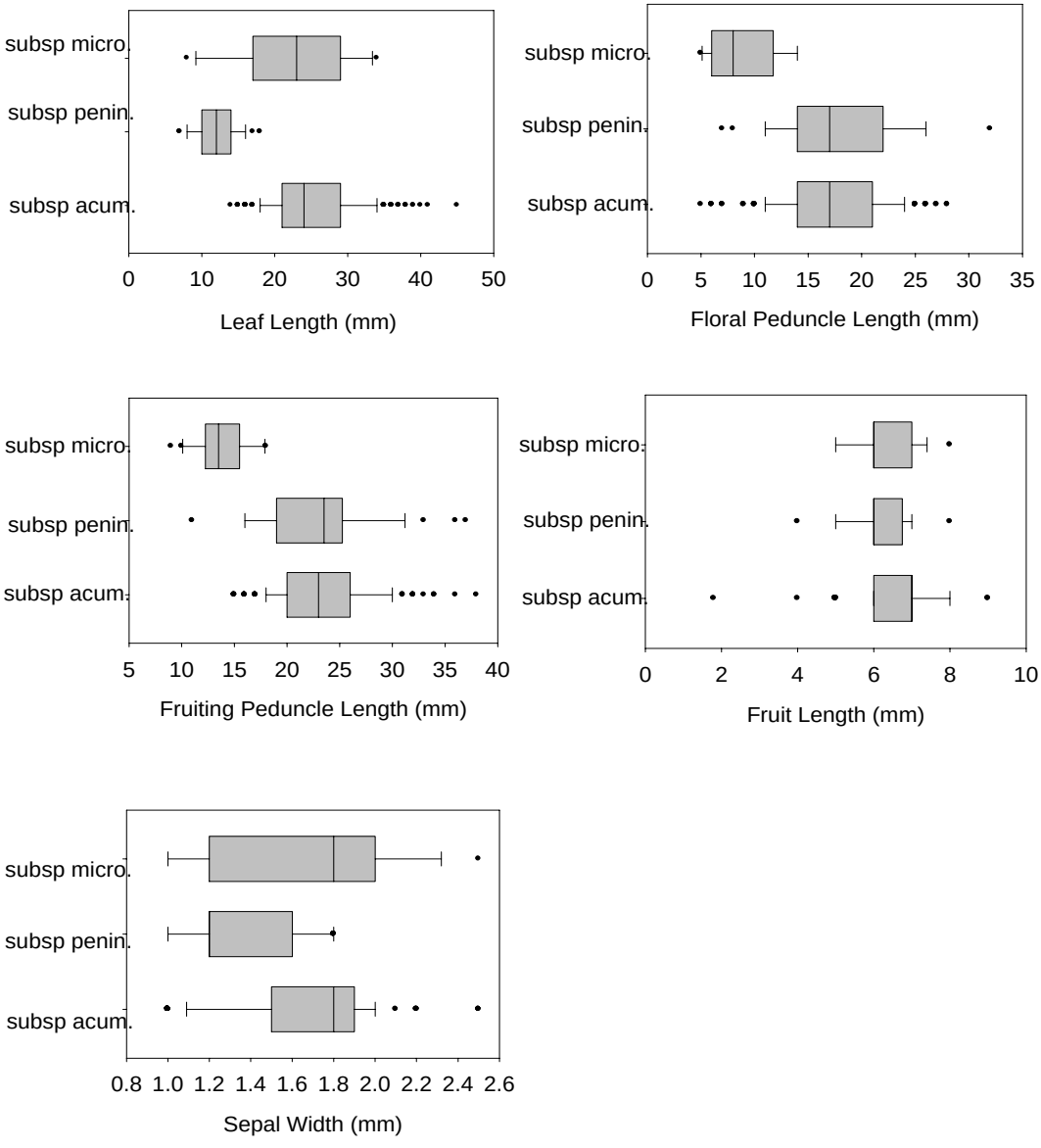
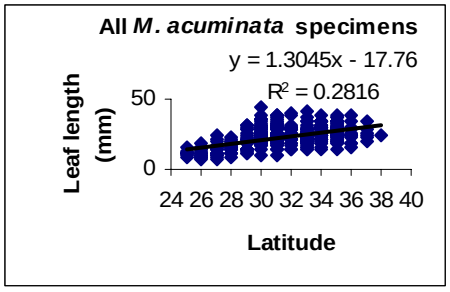
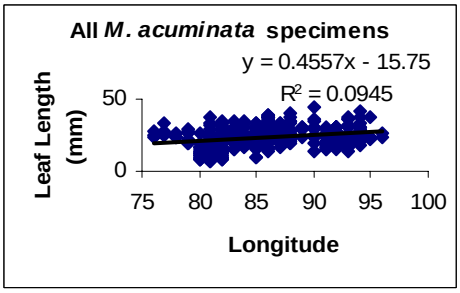
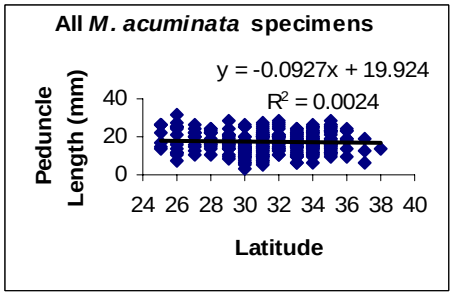
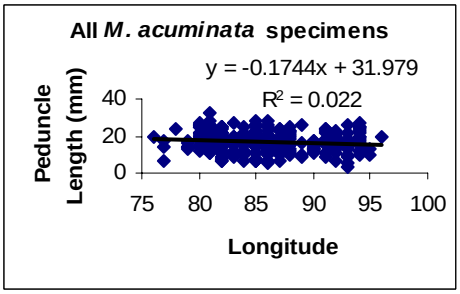


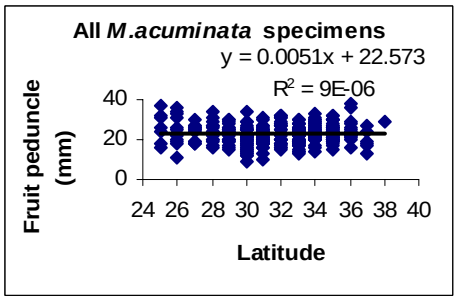
Figure 3. Linear Regression Analysis of all *M. acuminata* specimens showing latitudinal and longitudinal associations of characters.

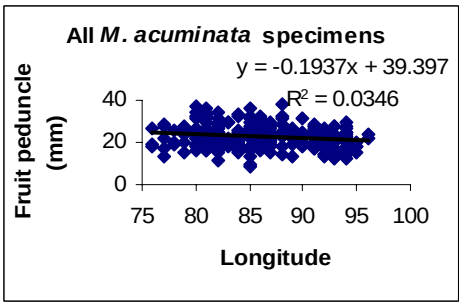


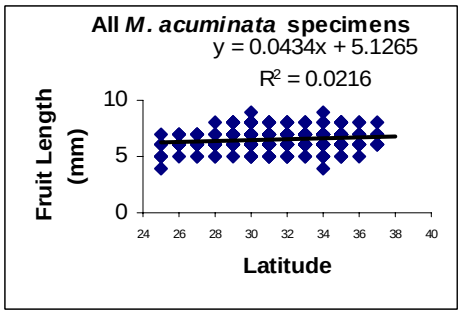












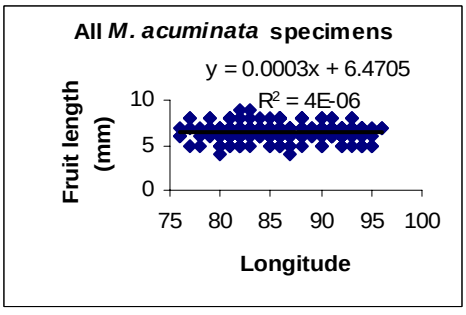
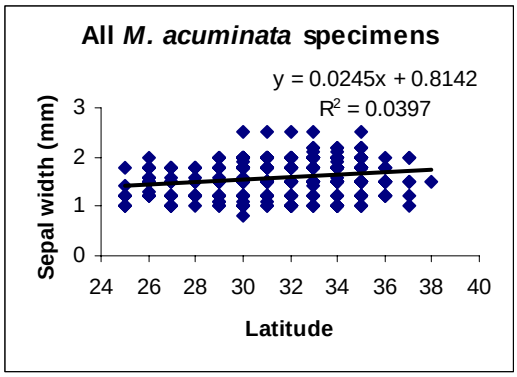


Fig 3 continued



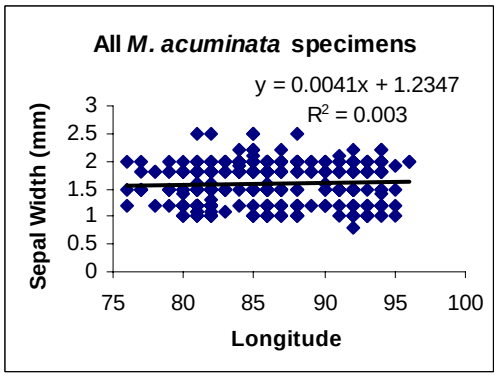


Figure 4. Two dimensional scatter plot of Cannonical Discriminant Function Analysis of morphological characters of *M. acuminata* compared to Latitude and Longitude.

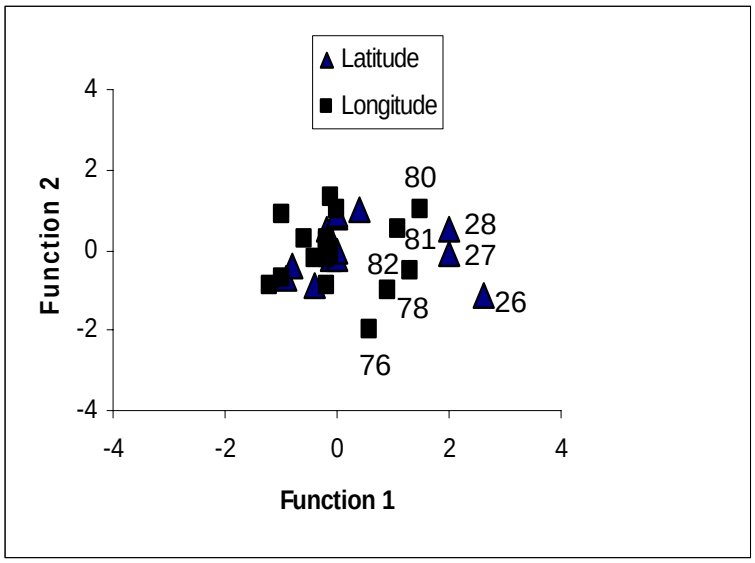


Figure 5. Two dimensional scatter plot of Cannonical Discriminant Function Analysis of morphological characters of *M. acuminata* compared to five biogeographic regions (1 = Florida Peninsular, 2 = Eastern Coastal Plains, 3 = Central Coastal Plains, 4 = West Coastal Plains, 5 = Northeastern region of Fall Line.

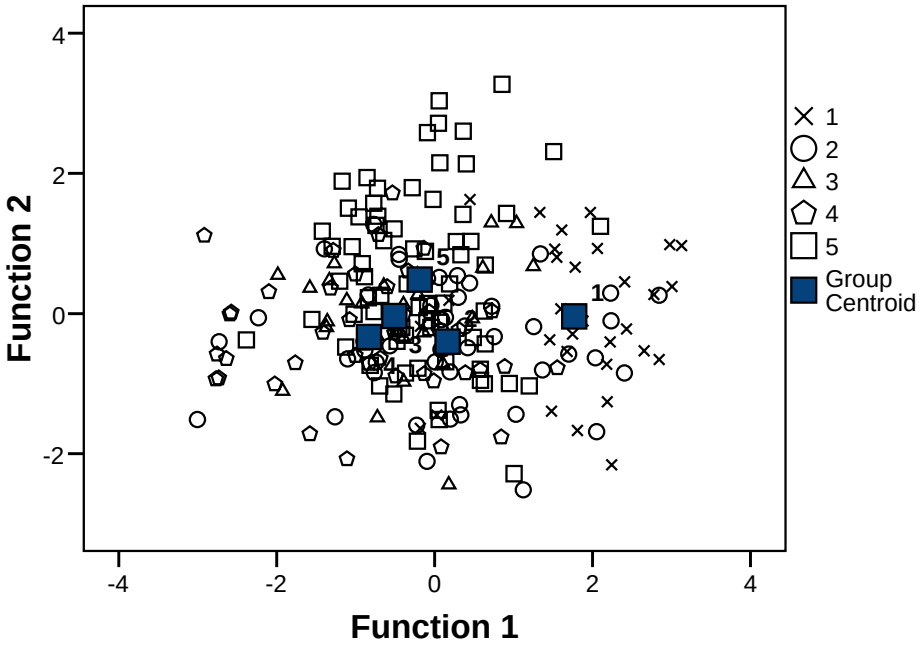


Figure 6. Two dimensional scatter plot of Cannonical Discriminant Function Analysis of morphological characters of *M. acuminata* compared to subspecies;

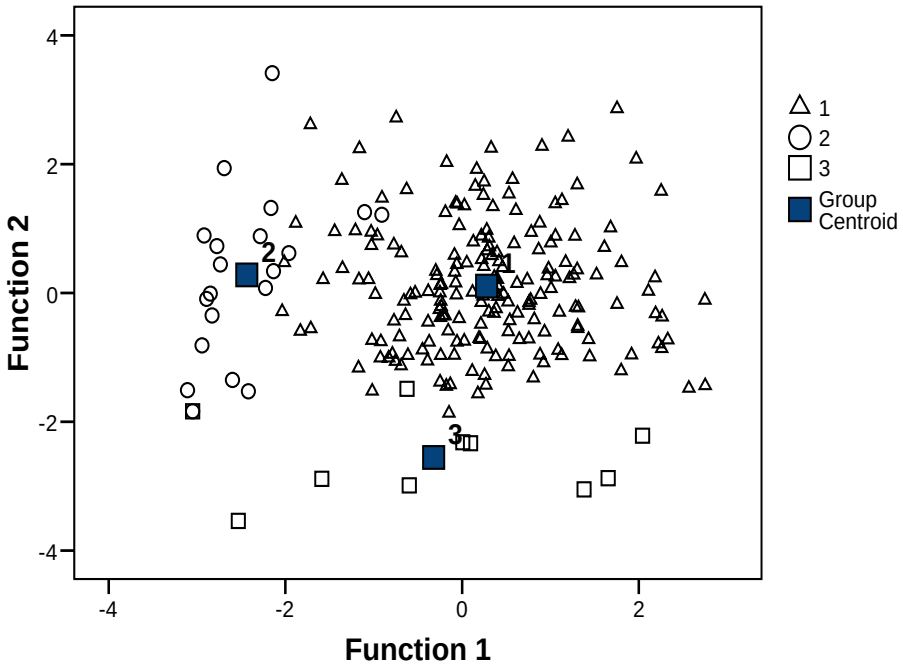
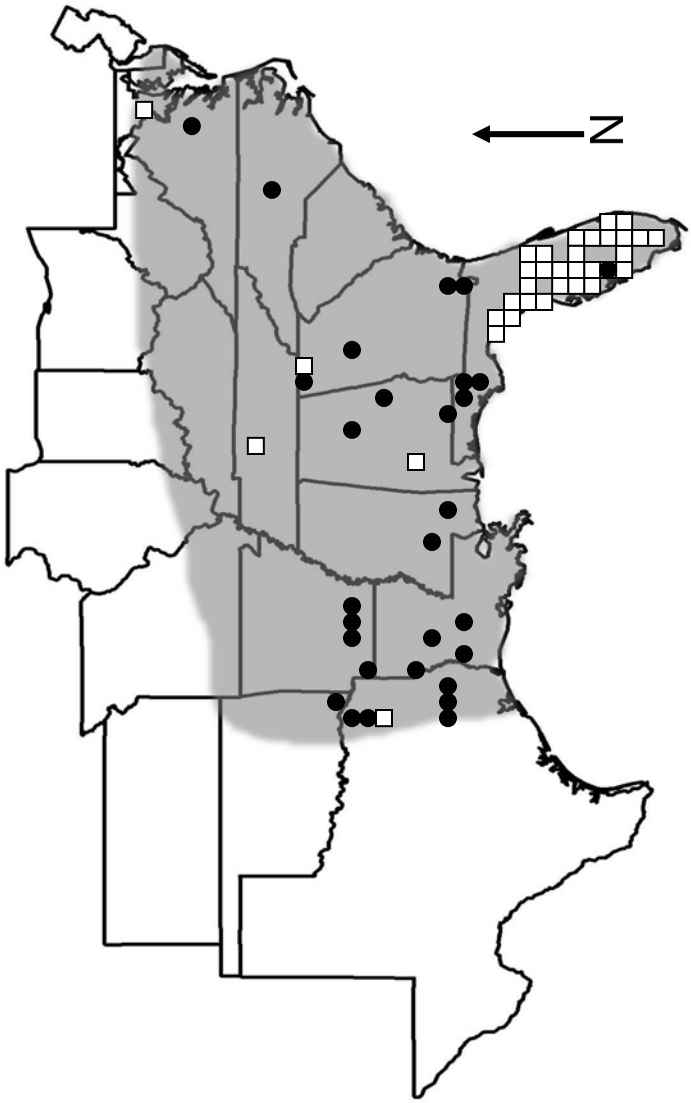


Fig. 7. Map showing distribution of subspecies *peninsularis*(squares) and *microphylla* (circles) within the range distribution of the complex. Shaded region denotes distributional range of subspecies *acuminata*.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Appendix 3-1. Information on herbarium and field specimens examined for the morphological study of *M. acuminata* complex in southeastern USA.

Mecardonia acuminata USA.

Alabama: Autauga Co., Moore 821(VDB); Barbour Co., Kral 33211(VDB); Charleston, Backman 93300 (MO); Choctaw Co., Causey 2089 (VDB); Cleburne Co., Kral 44910 (MO, VDB); Colbert Co., Webb 2225 (VDB); Covington Co., Duncan 14174 (GA); Covington Co., Elisens 1057 (OKL); Covington Co., Kral 88909 (VDB); Covington Co., MacDonald and Warren 13028 (VDB); Crenshaw Co., Diamond 12650 (VDB); Dale Co., Kral 54448 (VDB); Elmore Co., Kral 36612 (VDB); Elmore Co., McDaniel and Haynes 24314 (VDB); Fayette Co., Kral 48659 (MO, VDB); Franklin Co., Ahedor 112 (OKL); Franklin Co., Baskin et al., 516 (VDB); Franklin Co., Webb 4220 (VDB); Geneva Co., Kral 90297 (VDB); Green Co., Whitehouse 24382 (BRIT); Hale Co., Maginness 48 (VDB); Houston Co., MacDonald 3521(VDB); Jackson Co., Jones 7223 (VDB); Jackson Co., Webb 4840 (VDB); Lamar Co., Kral 66549 (VDB); Lawrence Co., 113 (OKL); Lee Co., Allison 2534 (GA); Lee Co., Kral 62508 (VDB); Marshall Co., Golden et al. (VDB); Monroe Co., Kral 32831(BRIT, VDB); Montgomery Co., Kral 41562 (VDB); Morgan Co., Webb and Dennis 3626 (VDB); Morgan Co., Whetstone and Atkinson 3439 (VDB); St. Clair Co., Kral 69566 (VDB); Sumter Co., Jones 13367 (VDB); Sumter Co., Kral 257 (FLAS); Sumter Co., Kral 257 (FLAS); Sumter Co., Kral 37025 (VDB); Talladega Co., Hood 302 (FLAS); Tuscaloosa Co., Thomas et al., 885 (VDB);

Arkansas: Ashley Co., Hamburg 16381A (MO); Ashley Co., Thomas 94971 (BRIT, FLAS, GA, MO); Ashley Co., Delmaree 16381A (BRIT); Cleveland Co., Thomas and

Sundell 163066 (VDB); Bradley Co., Delmaree 18327 (BRIT); Bradley Co., Delmaree 23854 (BRIT); Bradley Co., Delmaree 24646 (BRIT); Calhoun Co., Delmaree 22708 (BRIT, MO); Calhoun Co., Delmaree 22673 (BRIT); Calhoun Co., Delmaree 22506 (BRIT); Clay Co., Christ 2260766 (MO); Cleveland Co., Sundell and Ethridge 9135 (VDB); Drew Co., Demaree 24606 (GA); Drew Co., Monticello 24659 (GA); Drew Co., Delmaree 18281 (BRIT); Drew Co., Delmaree 18519 (BRIT); Drew Co., Delmaree 24566 (BRIT); Drew Co., Delmaree 24606 (BRIT); Drew Co., Delmaree 24659 (BRIT); Grant Co., Thomas et al., 173341 (BRIT); Hemptead Co., Moore 480481 (BRIT); Hot Spring Co., Delmaree 46683 (BRIT); Hot Spring Co., Delmaree 63026 (BRIT); Hot Spring Co., Scully 1853 (BRIT); Jefferson Co., Delmaree 24083 (BRIT); Lafayette Co., Delmaree 62985 (BRIT, VDB); Lee Co., McDaniel 1250 (BRIT); Little river Co., Tucker 10566; Logan Co., Delmaree 16015 (BRIT); Miller Co., Whitehouse 20315 (BRIT); Montgomery Co., Delmaree 34308 (BRIT); Ouachita Co., Thomas et al., 163315 & CD-593 (VDB); Perry Co., Delmaree 20151 (BRIT); Pope Co., Hightower (BRIT); Pope Co., Delmaree 19889 (BRIT); Woodruff Co., Delmaree 57266 (BRIT);

District of Columbia: Jones 1225 (Univ. of Georgia); Georgetown, Hermann 10782 (MO);

Florida: Alachua Co., Easley 740 (GA); Alachua Co., Dunn 322 (FLAS); Alachua Co., Tan 119 (FLAS); Alachua Co., Weber and West 21931 (FLAS); Beach Co., McCart 10895 (BRIT); Bay Co., Perkins and Nelson 396 (FLAS); Broward Co., McDaniel 9142 (FLAS); Broward Co., Will and Ward 87607 (FLAS); Calhoun Co., Elisens 1058 (OKL); Calhoun Co., Godfrey 60305 (BRIT); Calhoun Co., Hood 2702 (FLAS); Charlotte Co., Beckner 1734 (FLAS); Charlotte Co., Kral 7504 (FLAS); Charlotte Co., Krall 11978

(VDB); Charlotte Co., Murrill and Arnold 33955 (FLAS); Citrus Co., Baltzell and Zomlefer 2083 (FLAS); Citrus Co., Cooley et al., 6065 (GA); Citrus Co., Elisens 1061 (OKL); Citrus Co., Scanlon and Matthews 80 (FLAS); Citrus Co., West and Arnold 35434 (FLAS); Collier Co., 2474 (FLAS); Collier Co., Hill 122555 (MO); Collier Co., Hill 122555 (MO); Columbia Co., Herring and Herring 455 (FLAS); Dade Co., Avery 605 (FLAS); Craighead and Arnold 66609 (FLAS); Dade Co., Craighead and Arnold 72952 (FLAS); Dade Co., Herndon 929 (FLAS); Dade Co., Kral 18194 (VDB); Dade and Monroe Cos., Dade Co., Small et al., 6972 (FLAS); Dade Co., Small et al., 5972 (FLAS, MO); Dade Co., O'Neill 21932 (FLAS); DeSoto Co., Schallert 814 (FLAS); Dixie Co., Godfrey 56019 (BRIT, VDB); Dixie Co., West and Arnold 27268 (FLAS); DeSoto Co., Schallert 814 (FLAS); Dixie Co., West and Arnold 27268 (FLAS); Escambia Co., Ford and West 5586 (FLAS); Flagler Co., Slaughter 13869 (BRIT); Gadsden Co., Henderson 92-502 (MO); Gadsden Co., Henderson 96-752 (MO); Jacksonville, Curtiss 5170 (MO); Gadsden Co., Henderson 96-752 (MO); Gilchrist Co., Arnold 210 (FLAS); Hardee Co., Kirk and Arnold 38966 (FLAS); Hernando Co., Cooley et al., 6033 (GA); Curtiss 5170 (GA); Palm Beach Co., Cassen 463 (GA); Hendry Co., Eyles 6806 (GA); Hendry Co., Curtis 1868 (GA); Hendry Co., D'Arcy 1367 (FLAS); Hendry Co., McCart 10602 (BRIT, FLAS); Hernando Co., Baltzell and Judd 9531 (FLAS); Highlands Co., Brass 15366 (FLAS); Highlands Co., Garrett 50762 (FLAS); Hillsborough Co., O'Neil 21928 (FLAS); Hillsborough Co., Lakela 24011 (FLAS); Holmes Co., Godfrey 59008 (MO); Indian River Co., Baltzell and Judd 10786 (FLAS); Jackson Co., Garland 362 (GA); Jackson Co., Garland 362 (FLAS); Jackson Co., West 21966 (FLAS); Jacksonville, Curtiss 5170 (MO); Lake Co., Miller and Perkins 257 (FLAS); Lake Co., Robinson 1192

(FLAS); Lee Co., Hood 3422 (FLAS); Lee Co., Kral 57261 (VDB); Lee Co., Kral 64716 (VDB); Lee Co., Murrill and Arnold 210 (FLAS); Lee Co., Schallert 814 (BRIT); Leon Co., Henderson 93-331 (MO); Levy Co., Arnold 210 (FLAS); Levy Co., Amoroso and Judd 878 (FLAS); Levy Co., Elisens 1064 (OKL); Levy Co., Greenberg 132 (FLAS); Levy Co., Tiley and Notis 2332 (FLAS); Levy Co., West and Arnold 27010 (FLAS); Liberty Co., Elisens 1059 (OKL); Manatee Co., West 21967 (FLAS); Manatee Co., Cuthbert 1382 (FLAS); Manatee Co., Ray et al., 10162 (VDB); Marion Co., Baltzell and Judd 7700 (FLAS); Monroe Co., Porter 12434 (BRIT); Martin Co., Baltzell and Judd 10235 (FLAS); Marion Co., West and Arnold 35348 (FLAS); Okaloosa Co., Stone and Bradley 3434 (MO); Okaloosa Co., West 61070 (FLAS); Okeechobee Co., Correll 51747 (MO); Okeechobee Co., McCart 10,763 (BRIT, FLAS); Okeechobee Co., McCart 10763 (BRIT); Osceola Co., Hall et al., 557 (FLAS); Orange Co., Beckner 1800 (FLAS); Orange Co., West 21965 (FLAS); Palm Beach Co., Cassen 563 (FLAS); Palm Beach Co., McCart 10,895 (FLAS); Palm Beach Co., McCart 10,911 (BRIT, FLAS); Palm Beach Co., West and Arnold 34210 (FLAS); Palm Beach Co., Kral 5654 (BRIT); Palm Beach Co., Palm Beach Co., Kral 5654 (BRIT); Palm Beach Co., McCart 10911(BRIT); Pasco Co., Baltzell 7742 (FLAS); Pinellas Co., Carter 2339 (VDB); Pinellas Co., West 257 (FLAS); Polk Co., Baltzell and Zomlefer 9762 (FLAS); Polk Co., Hood 4184 (FLAS); Polk Co., Hood 4184 (FLAS); Putnam Co., Baltzell and Hall 1160 (FLAS); Putnam Co., Ionta and Marks 78 (FLAS); Santa Rosa Co., Burkhalter and Hall 5604 (FLAS); Saratosa Co., 257 (FLAS); Seminole Co., Scudder and Beckner 0433 (FLAS); St. Johns Co., Ward and Myint 2108 (FLAS); St. Lucie Co., Garland 899 (FLAS); Sumter Co., Sargent 6404 (BRIT); Taylor Co., Duncan 14003 (GA); Taylor Co., Edwards and Ionta 60 (FLAS);

Taylor Co., Godfrey 60380 (BRIT); Union Co., Penneys 1135 (FLAS); Union Co., Cherry et al., 85 (FLAS); Volusia Co., Ray et al., 10835 (BRIT); Volusia Co., Robinson 22959 (FLAS); Wakulla Co., Anderson 4353 (BRIT); Walton Co., 257 (FLAS); Walton Co., Hood 2999 (FLAS); Washington Co., Hood 2876 (FLAS); Washington Co., Hood 2877 (FLAS);

Georgia: Appling Co., Nordman (GA); Baker Co., Duncan 4114 (GA, MO); Bartow Co., Greear 64403 (GA); Bryan Co., Carter et al., 10330 (GA, VDB); Biscayne Bay Palmer 353 (MO); Calhoun Co., Thorne 6495 (GA); Catoosa Co., Cronquist 5612 (BRIT, GA, MO); Catoosa Co., Duncan 13213 (GA); Catoosa Co., Kral 537 (VDB); Charlton Co., Blake 67708 (GA); Colquitt Co., Faircloth 4841 (GA); Columbia Co., Duncan 10248 (GA); Crawford Co., Payne and Payne 7546 (GA); Cutler, Small and Carter 1161140 (MO); Decatur Co., Duncan 13993 (GA); Echols Co., Carter 4451 (FLAS, GA); Elbert Co., Coile and Coile 1190 (GA); Elbert Co., Dunn 1268 (FLAS); Dekalb Co., McDowell and Venard M-482 (GA); Floyd Co., Duncan 13120 (GA); Floyd Co., Ware Sr. 31 (GA); Gordon Co., Moore et al., 4270 (GA); Grady Co., Faircloth 1611 (MO); Grady Co., Faircloth 2988 (GA, MO); Hancock Co., Allison and Duncan 30866 (GA); Hart Co., Credle 2534 (GA); Hart Co., Credle 2323 (GA); Hart Co., Duncan 7820 (GA); Heard Co., Allison 2508 (GA); Jasper Co., Duncan 21559 (GA); Jefferson Co., Pyron and McVaugh 1238 (GA); Lake Co., Nash 688 (MO); Lee co., Harper et al., 17165 (GA); Lee Co., Hitchcock 250 (MO); Liberty Co., Carter and Lusk 10407 (GA); Long Co., Bozeman 1754 (GA); Long Co., Duncan 7110 (GA); Madison Co., Duncan 11619 (GA); Marion Co., Orzell and Bridges 15111 (FLAS); Meriwether Co., Patrick et al., 2992 (GA); McIntosh Co., Angerman 7546 (GA); Muscogee Co., Allison 2549 (GA); Newton

Co., Allison 2368 (GA); Oconee Co., Seward 1396 (GA); Orange City, Hood 850428 (MO); Peach Co., Payne and Payne (GA); Seminole Co., Boring et al., 3144 (GA); Sumter Co., Harper 637 (MO); Sumter Co., Norris 6363 (GA); Tattnall Co., Zebryk 0601 (GA); Tattnall Co., Zebryk 0730 (GA); Walker Co., Coile and Jones 24361 (GA); Wheeler Co., Carter 6355 (VDB); Wilcox Co., Elisens 1066 (OKL); Wilkes Co., Allison 2776 (GA);Wilkes Co., Fitzgerald 504 (GA); Whitfield Co., Gioia 47 (GA);

Kentucky: Calloway Co., Landon et al., 4494 (VDB);

Louisiana: Allen Pa., Ahedor 101 (OKL); Allen Pa., Shinners 21518 (BRIT); Allen Pa., Vincent 3826 (BRIT); Beauregard Pa., Ahedor 102 (OKL); Beauregard Pa., Shinners 21574 (BRIT); Bienville Pa., Slaugther 1419 (GA); Bossier Pa., Shinners 24499 (BRIT); Caldwell Pa., Shinners 21918 (BRIT); Caldwell Pa., Thomas 108,595 (FLAS); Caldwell Pa., Thomas 125, 216 (MO); Catahoula Co., Thomas and French 41195 (BRIT); Covington Pa., Arsene 11167 V; DeSoto Pa., Vincent 4173(BRIT); Evangeline Pa., Thieret 10181(VDB); Evangeline Pa., Thieret 16420 (VDB); Evangeline Pa., Vincent 4131 (GA); Evangeline Pa., Thieret 16420 (BRIT); Elmore Co., Kral 36612 (GA); Grant Pa., Shinners 21332 (BRIT); Jackson Pa., Miller 125 (MO); Jefferson Davis Co., Vincent 3773 (FLAS); LaSalle Pa., Thomas and Laird 30048 (VDB); Lincoln Pa., Kral 15790 (VDB); Natchitoches Pa., Kral 16188 (VDB); Natchitoches Pa., Thomas et al., 108, 449 (MO); Ouachita Pa., Thomas et al., 93755 (MO); Red River Pa., Shinners 28745 (BRIT); Sabine Pa., Shinners 21623(BRIT); Sabine Pa., Vincent 3850 (MO); St. Landry Pa., Vincent 3891 (FLAS); St. Tammany Pa., Allen 9610 and Vincent 2964 (FLAS); St. Tammany Pa., Allen 9610 and Vincent 2964 (MO); St. Tammany PA., Elisens 1053 (OKL); St. Tammany Pa., Rylander 7 (BRIT); St. Tammany Pa., Rylander 31 (FLAS);

Tensas Pa., Thomas 101, 071 (MO); Union Pa., Shinners 24606 (BRIT); Washington Pa., Allen et al., 698 (BRIT); Washington Pa., Allen et al., 2698 (FLAS); Washington Pa., Allen 9660 and Vincent 3015 (MO); Washington Pa., Ewan 19427; Winn Pa., Vincent and Allen 195 (FLAS); Washington Pa., Vincent et al., 2698 (VDB); Webster Pa., Shinners 21781; West Feliciana Pa., Allen et al., 9535 (BRIT); Winn Pa., Elisens 1047 (OKL); Winn Pa., Thomas et al., 56061(BRIT);

Maryland: Montgomery Co., Iltis 1066 (BRIT); Mongomery Co., Painter 1041 (MO);

Mississippi: George Co., Elisens 1056 (OKL); George Co., Shinners 28847 (VDB); Itawamba Co., Kral 74882 (VDB); Kemper Co., McDaniel and Daugherty 32036 (VDB); Lamar Co., Jones Jr. 2500 (GA); Madison Co., Chapman et al., 18035 (GA); Marion Co., Jones et al., 20276 (VDB); Marion Co., Parker et al., 20276 (GA); Oktibbeha Co., Bryson 8291 (GA,VDB); Scott Co., Chapman et al., 17832 (FLAS); Scott Co., Jones et al., 17744 (VDB); Simpson Co., Jones 14049 (GA); Smith Co., Jones 19154 (BRIT); Star, Tracy 8713 (BRIT); Tallahatchie Co., Temple 6802 (GA); Tishomingo Co., Ray Jr. 7382 and Gleason 1960 (VDB);

Missouri: Butler Co., Hudson 182 (MO); Butler Co., Hudson 287; Butler Co., Hudson 325; Butler Co., Rowan 1066; Carter Co., Hudson 791 (MO); Howell Co., Summers 3815; Howell Co., Summers et al., 9517; Howell Co., Summers 10092-A; Howell Co., Summers 10085; Howell Co., Summers 3420; Howell Co., Summers 7078 (MO); Jasper Co., Palmer 799; Jasper Co., Palmer 840; Jasper Co., Palmer 799 (MO); Jasper Co., Palmer 2816; Jasper Co., Palmer 3437 (MO); Jasper Co., Palmer 26294; Jasper Co., Palmer 2816 (MO); Howell Co., Summers 6336 (MO); Mississippi Co., Steyermark 9077; Scott Co., Holmes 775; Stoddard Co., Brant 4794 (MO);

North Carolina: Bertie Co., Radford 5967 (GA); Chatham Co., Fox and Godfrey 3146 (BRIT); Durham Co., Blonquist 16771(BRIT); Franklin Co., Ingle 2039 (VDB); Granville Co., Batson 1217 (BRIT); Jones Co., Radford 40053 (VDB); Lee co., Beard 703 (BRIT); Mongomery Co., Oosting 1870 (FLAS); Orange Co., Nesom 124 (BRIT); Robeson Co., Terrell 2991 (FLAS); Rowan Co., Small and Heller 345 (MO); Union Co., Ahles 33912 (BRIT);

Oklahoma: Bryan Co., Taylor and Taylor 3446 (OKL); McCarty GRU0773 (OKL); McCurtain Co., Hoagland and Benesh RSGS453 (OKL); McCurtain Co., Waterford 10423 (BRIT); Muskogee Co., Bebb 4987 (OKL); Muskogee Co., Johnson et al., GRU0080 (OKL); Muskogee Co., Proctor and McCarty GRU0708 (OKL); Muskogee Co., Waterford 10297 (BRIT);

South Carolina: Abbeville Co., Credle 3037 (GA); Aiken Co., Angerman (VDB); Allendale Co., Bell 5149 (GA); Berkeley Co., Merello and Noyes 417 (MO); Berkeley Co., Myers 16 (GA); Columbia Richland Co., Philson 15118 (GA); Diken Co., Eggert 93299 (MO); Mebburtis 93296; 932297 (MO); McClellanville, PVS 1063199 (MO); St. Helen Island Cuthbert 21924 (FLAS);

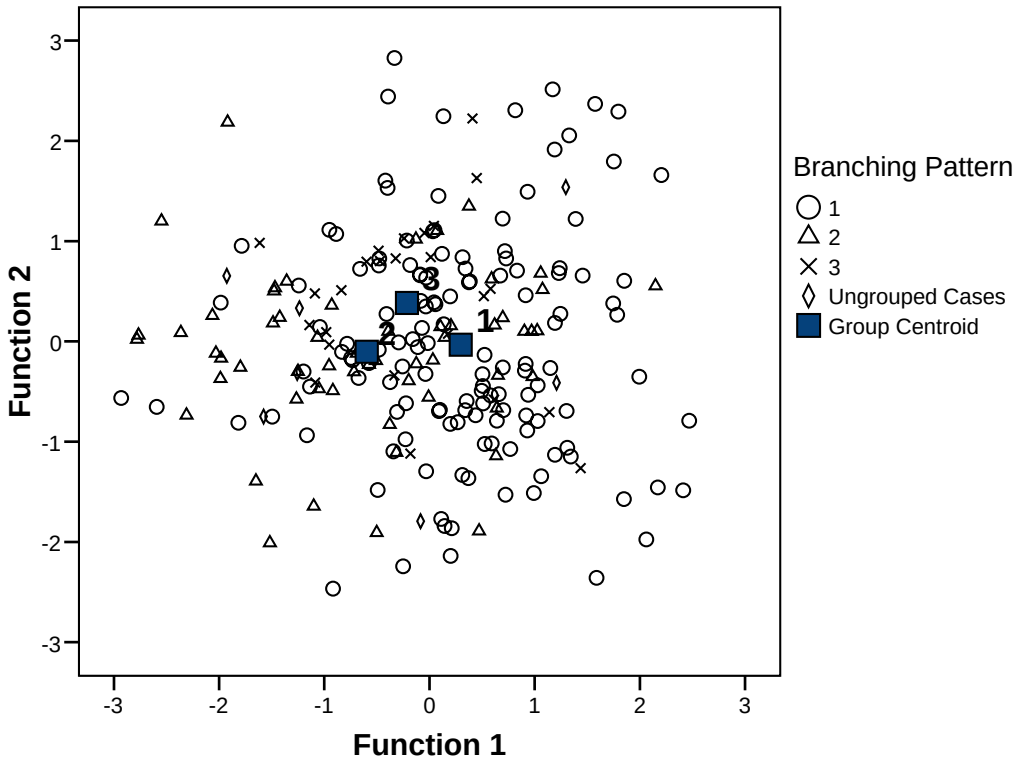
Tennessee: Bedford Co., Ahedor 107 (OKL); Benton Co., Shaver 8424 (GA); Benton Co., Shaver 7546; Bedford Co., Kral 40740 (BRIT, MO, VDB); Coffee Co., Baskin et a., 30 (VDB); Coffee Co., Horn and Kral 375 (VDB); Coffee Co., Kral 26027 (VDB); Coffee Co., Kral 32289 (BRIT); Coffee Co., Kral 32289 (VDB); Coffee Co., Kral 40690 (GA, MO, VDB); Coffee Co., Shaver 8425 (GA); Coffee Co., Somers and Collins 1512 (VDB); Davidson Co., Guthrie 594 (VDB); Davidson Co., Franklin et al., 224)VDB); Franklin Co., Shaver 8526 (GA); Hardin Co., Kral 76872 (VDB); Hardin Co., Shaver

9902 (VDB); Lawrence Co., Shaver 9925 (BRIT, VDB); Lewis Co., Kral 36201 (VDB); Macon Co., Kral 79582 (VDB); Madison Co., Webb et al., 2326 (VDB); Marshall Co., Ahedor 105 (OKL); Maury Co., Collins 4527 (VDB); Maury Co., Ahedor 106 (OKL); McNary Co., Svenson 4333 (BRIT); Meigs Co., Sharp and Underwood 2323 (GA); Moore Co., Kral 40652 (GA, VDB); Montgomery Co., Chester 4452 (VDB); Montgomery Co., Chester and Wofford 1781 (BRIT); Perry Co., Kral 68922 (VDB); Rutherford Co., Ahedor 104 (OKL); Rutherford Co., Ahedor 108 (OKL); Rutherford Co., Ahedor 109 (OKL); Rutherford Co., Ahedor 111 (OKL); Rutherford Co., Kral 29206 (VDB); Rutherford Co., Shaver 7546 (VDB); Rutherford Co., Pyne 93-225 (VDB); Sequatchie Co., Rogers 44181(VDB); Shelby Co., Heineke 2852 (MO); Wilson Co., Quarterman 4018 (VDB); Wilson Co., Rogers 44301 (BRIT); Wilson Co., Rogers 44301 (VDB); Wilson Co., Ahedor 110 (OKL); **Texas:** Anderson Co., Whitehouse 22385 (BRIT); Angelina Co., Shinners 26799 (BRIT); Cass Co., Shinners 24828 (BRIT); Cass Co., Whitehouse 17682 (BRIT); Cherokee Co., Lewis et al., 5505 (BRIT); Echols Co., Carter 4451 (VDB); Harrison Co., Cory 57753 (BRIT); Harrison Co., Fleetwood 12397 (BRIT); Harrison Co., Fleetwood 12407 (BRIT); Harrison Co., Lindheimer and Dapprich 7466 (BRIT); Jasper Co., Lundell 11817 (MO); Jasper, Tharp and Pennell 10574 (MO); Jasper Co., Tharp 2503 (MO); Leon Co., Correll 14178 (BRIT); Montgomery Co., Shinners 16592 (BRIT); Nacogdoches Co., Ahedor 103 (OKL); Nacogdoches Co., Nixon and Stransky 2864 (MO); Newton Co., Cory 49781 (BRIT); Newton Co., Cory 49783a (BRIT); Orange Co., Corbin 80 (BRIT); Polk Co., Shinners 25332 (BRIT); Red River Co., Taylor 10668 (BRIT); Red River Co., Robertson Co., Starbuck 906 (BRIT); Sabine Co., Whitehouse 16578 (BRIT); San Jacinto Co., Shinners 25326 (BRIT); Shelby Co.,

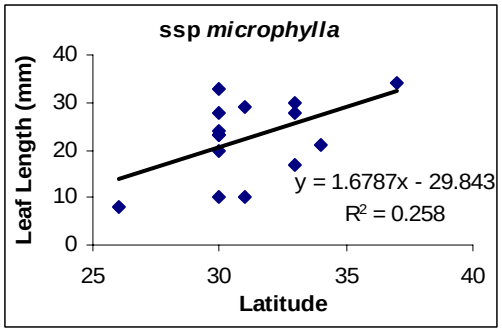
Whitehouse 16669 (BRIT); Whitehouse 20546 (BRIT); Smith Co., Fleetwood 12621(BRIT); Tattnall Co., Zebryk et al., 0601 (VDB); Titus Co., Ajilvsgi 7136 (BRIT); Titus Co., Ajilvsgi 7004 (BRIT); Titus Co., Amerson 980 (BRIT); Tyler Co., Cory 54894 (BRIT); Upshur Co., Shinners 16016 (BRIT); Van Zandt Co., Whitehouse 16450 (BRIT); Wilcox Co., Kral 81830 (VDB);

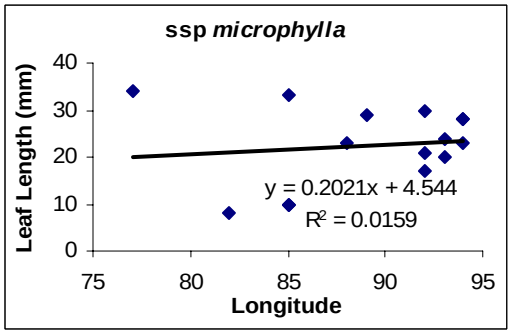
Virginia: Amelia Co., Lewis 2024 (BRIT); Henrico Co., Seymour and Svenson 3005299 (MO); King William Co., Bradley 24110 (GA); Matheus Co., Rothrock 93295 (MO); Nansemond Co., Hubricht B2676 (MO); New Kent Co., Soltis 548 (FLAS); Westmoreland Co., Iltis 359 (BRIT); York Co., Kirkman and Ware 815 (FLAS);

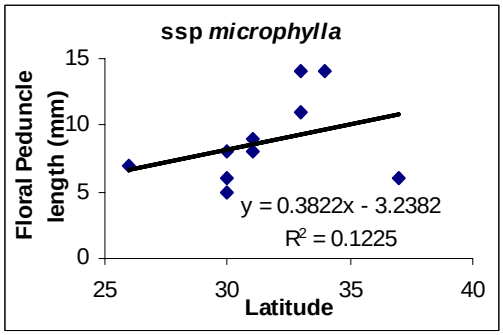
Appendix 3-2. Two dimensional scatter plot of Canonical Discriminant Function Analysis of morphological characters of *M. acuminata* compared to three branching patterns.

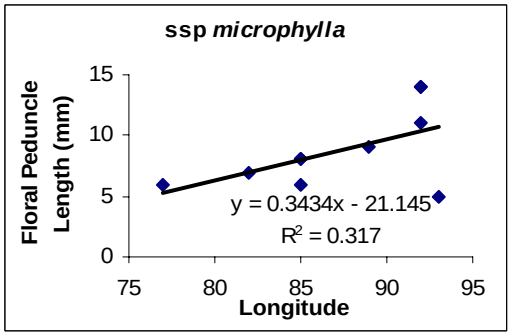


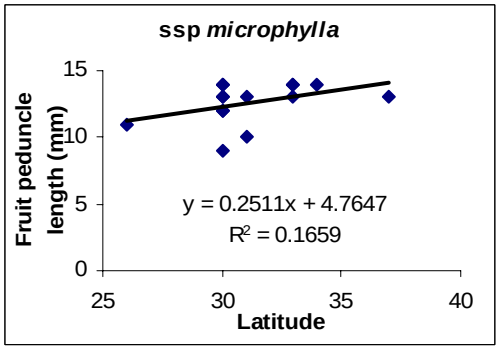
Appendix 3-3. Linear Regression Analysis of subspecies *microphylla* specimens showing latitudinal and longitudinal associations of characters.

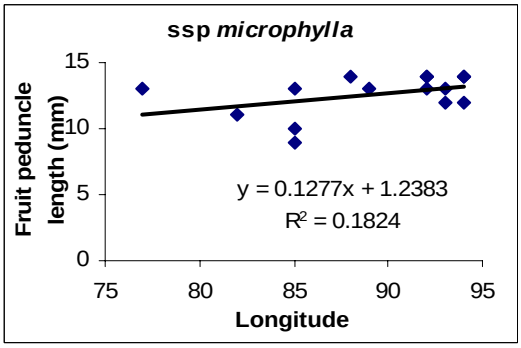


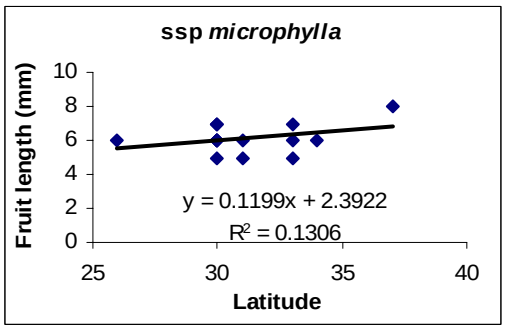


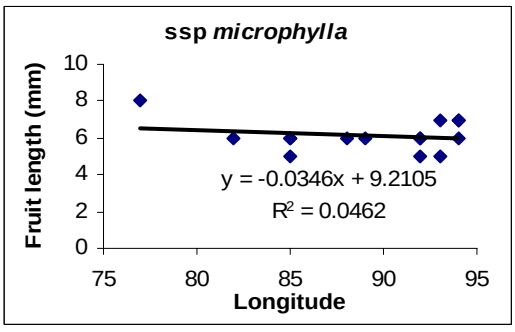












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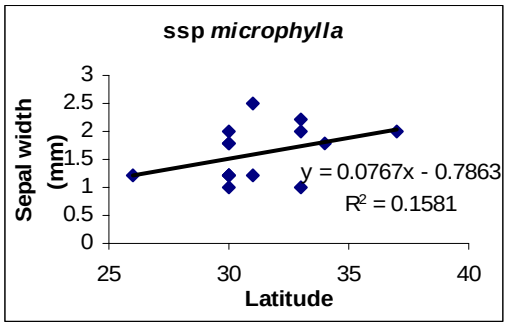
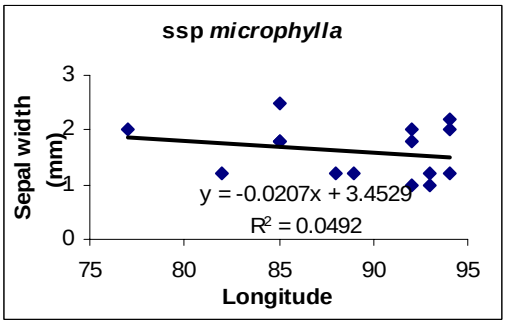
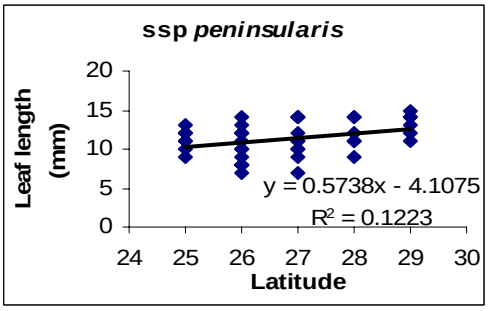
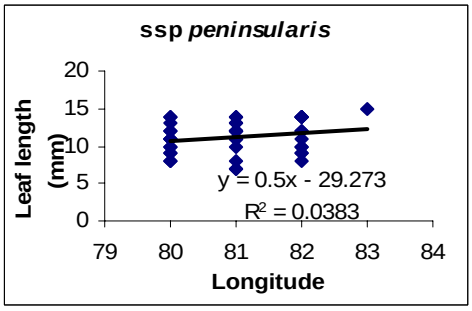
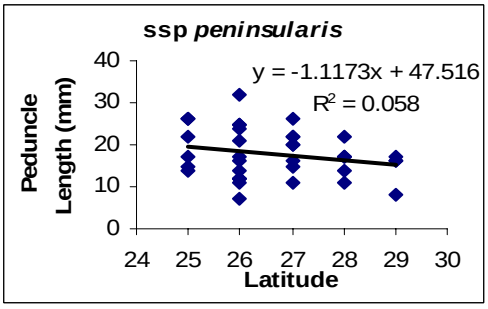


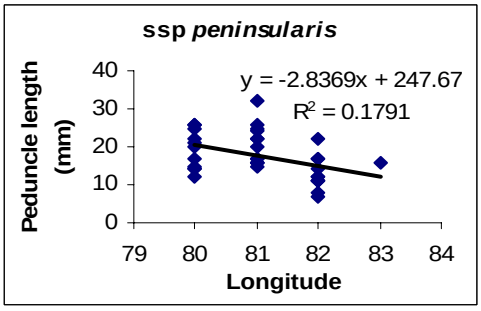


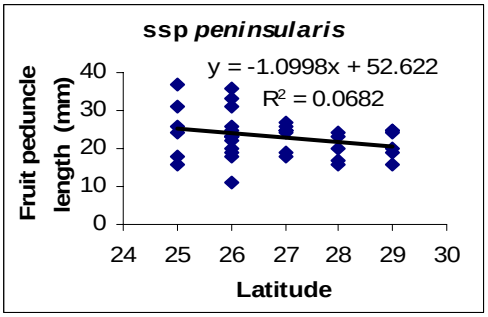
Appendix 3-4. Linear Regression Analysis of subspecies *peninsularis* specimens showing latitudinal and longitudinal associations of characters.

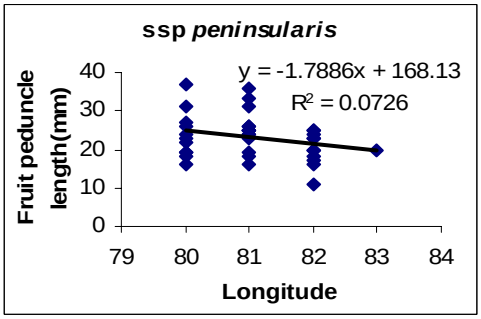


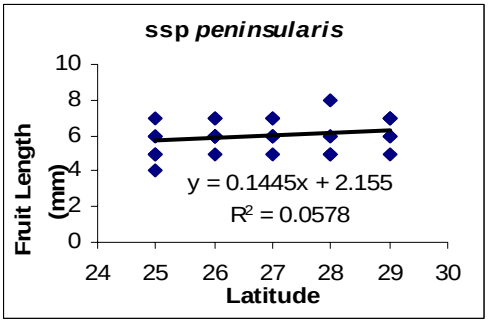


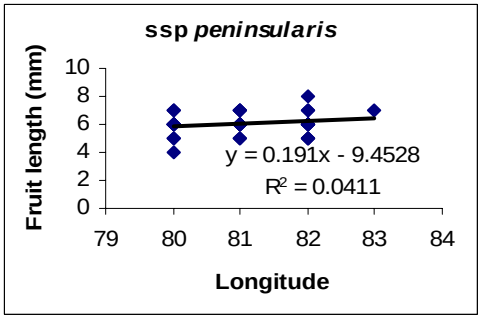












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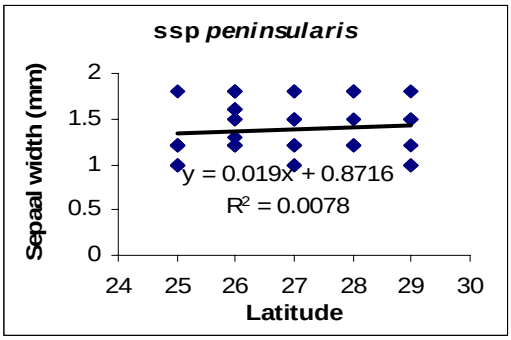
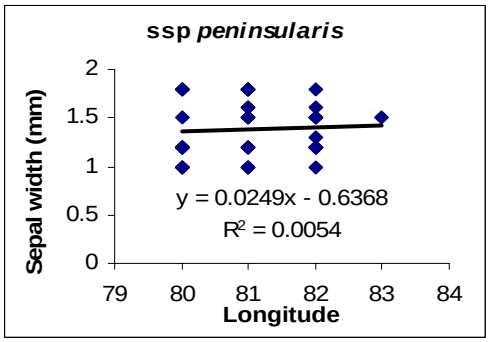
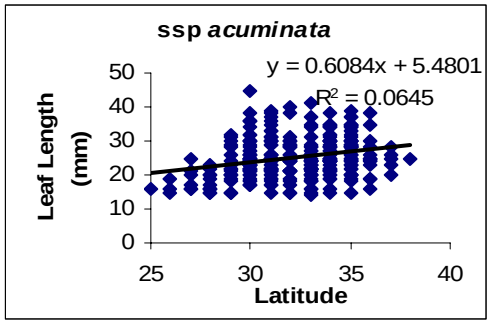
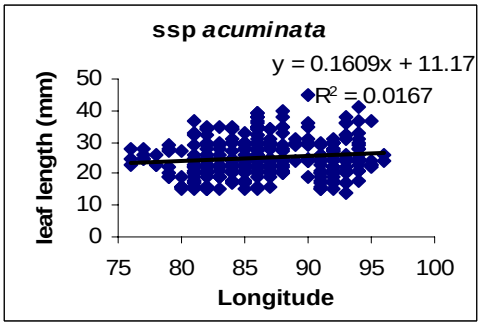
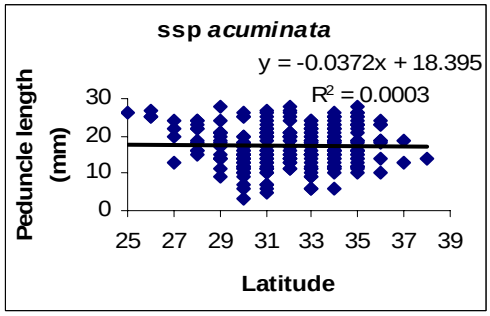


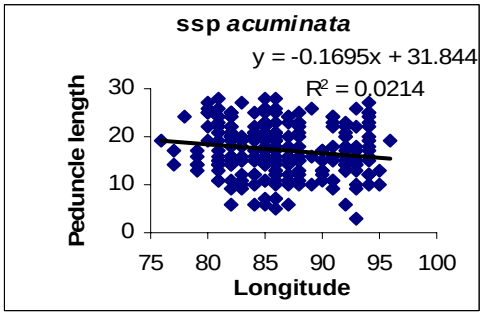


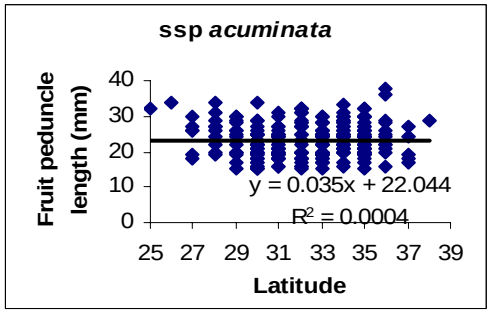
Appendix 3-5. Linear Regression Analysis of subspecies *acuminata* specimens showing latitudinal and longitudinal associations of characters.

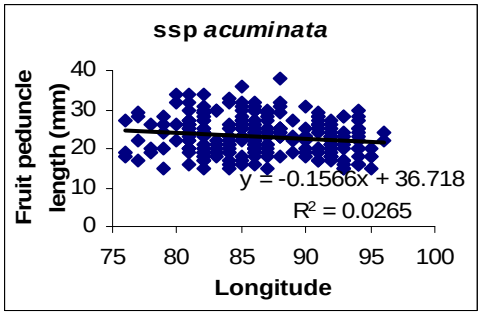


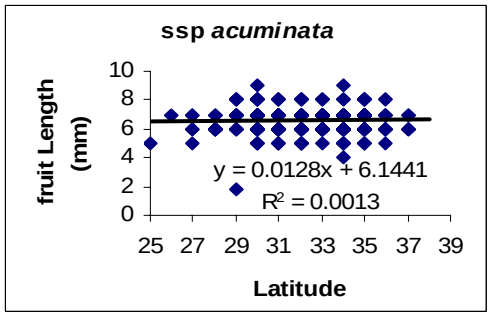


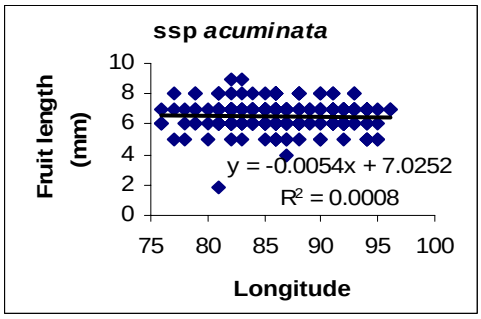












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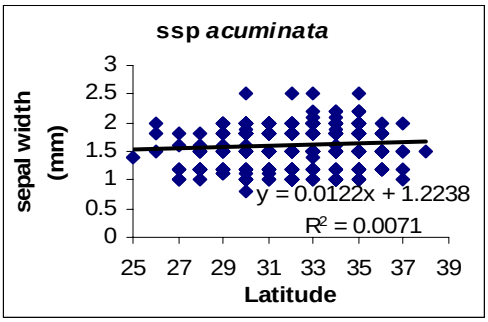

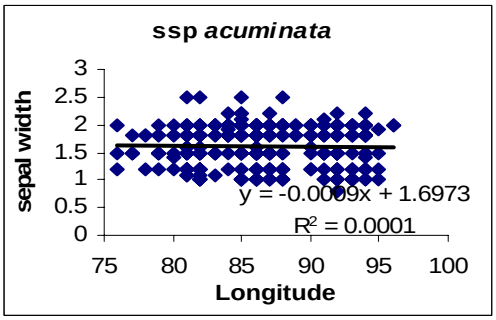

Appendix 3- 6. Table showing states and counties where subspecies *peninsularis* and *microphylla* were identified (counties in bold denote counties where both subspecies were identified)

Subspecies *peninsularis*		Subspecies *microphylla*	
State	County	State	County
Florida	Broward	Florida	Calhoun
	Charlotte		**Charlotte**
	Citrus		Jackson
	Dade		Washington
	Desoto	Alabama	Geneva
	Dixie		Lee
	Gilchrist		St. Clair
	Hardee	Mississippi	George
	Hendry		Lamar
	Hernando	Louisiana	Beauregard
	Highlands		Evangeline
	Indian River		Grant
	Lake	Texas	Newton
	Lee		Orange
	Levy		Polk
	Manatee		Red River
	Martin		SanJacinto
	Marion		Titus

Appendix 3- 6 continued.

	Okeechobe		Tyler
	Palm Beach	Virginia	King William
	Pasco	North Carolina	Chatham
	Pinellas	Georgia	Charlton
	Polk		**Catoosa**
	Seminole		Dekalb
	St. Lucie	Arkansas	Drew
	Taylor		Bradley
	Volusia		Calhoun
Georgia	**Catoosa**		Lafayette
Alabama	Wilcox	Oklahoma	McCurtain
(Maryland)	D.C.		
Tennessee	Rutherford		
Texas	Ushur		

LIST OF APPENDICES

Page