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BIOSYSTEMATICS OF NORTH AMERICAN SPECIES OF *NUTTALLANTHUS* (LAMIALES)

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By

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BIOSYSTEMATICS OF NORTH AMERICAN
SPECIES OF *NUTTALLANTHUS* (LAMIALES)

A DISSERTATION
APPROVED FOR THE DEPARTMENT OF
BOTANY AND MICROBIOLOGY

BY

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ABSTRACT OF DISSERTATION

The native North American toadflaxes comprise a morphologically variable complex of herbaceous plants that have been collectively recognized as a section of *Linaria* (*Linaria* section *Leptoplectron*) or a distinct genus (*Nuttallanthus* D. Sutton) and individually assigned to specific or varietal rank. High levels of intraspecific morphological variation and intergradation in the morphological characters used to distinguish among these taxa have led to diverse taxonomic treatments of the members of this group. This study represents the first use of enzyme electrophoresis to examine patterns of genetic variation in this species complex. Despite their wide geographic distribution and showy, fragrant flowers (seemingly adapted for insect pollination and outbreeding) the extent and distribution of genetic variation at the populational level was similar to that of narrowly distributed, autogamous species; a low level of detected heterozygotes suggested a high degree of inbreeding in these taxa. Field and greenhouse breeding-system studies indicated that individuals of all three species were entirely self-compatible and that the primary reproductive strategy of these taxa involved cleistogamy and self-pollination. No hand-performed cross pollinations among species resulted in the successful development of capsules and seeds, indicating that these species were reproductively isolated in areas of sympatry. Univariate and multivariate analyses of morphological characters measured from field-collected samples revealed significant morphological gaps among taxa and consistently separated samples into three primary clusters corresponding to the currently circumscribed species; strong and significant correlations between patterns of morphological and genetic variation were observed among populations and species. The observed morphological discontinuities separating

groups in this genus and the close correspondence of extensive morphological and genetic gaps among these taxa are concordant with the observed cross-incompatibility among species; these results strongly support the recognition of three species in North American *Nuttallanthus*. Due to similar genetic divergence values among species, phylogenetic relationships in *Nuttallanthus* remain unresolved. Patterns of genetic and morphological differentiation within and among these species suggests a long history of reproductive isolation due to biotic and paleogeographic barriers to gene flow.

CHAPTER 1.

GENETIC VARIATION AND REPRODUCTIVE BIOLOGY OF NORTH AMERICAN SPECIES OF *NUTTALLANTHUS* (LAMIALES)

ABSTRACT

The native North American toadflaxes comprise a morphologically variable complex of taxa that have been collectively recognized as a section of *Linaria* (*Linaria* section *Leptoplectron*) or a distinct genus (*Nuttallanthus* D. Sutton) and individually assigned to specific or varietal rank. To assess patterns of genetic variation within and among species, starch gel electrophoresis was employed to examine 15 scorable isozyme loci for 50 populations. Species are widely distributed geographically, occur in structurally-homogeneous populations ranging in size from one to thousands of individuals, and possess showy, fragrant flowers seemingly adapted for insect pollination and outbreeding. Despite these characteristics, the amount and apportionment of genetic variation within and among populations was similar to that of narrowly distributed, autogamous species. A low level of detected heterozygotes suggested a high degree of inbreeding. Genetic identities among species were quite low (ranging from 0.516 to 0.623) relative to intraspecific identity values (ranging from 0.819 to 0.936); qualitative differences among species were evident at several loci. Field and greenhouse breeding-system studies indicate high levels of self-fertilization; all individuals studied were entirely self-compatible and many chasmogamous flowers selfed prior to anthesis. Individuals of all three species commonly produced cleistogamous (obligately-selfing) flowers both early and late in the life cycle. No hand-performed cross pollinations among species resulted in the successful development of capsules and seeds. The low interspecific genetic identity estimates, high proportion of unique alleles (ranging from 4 to 9 per species), and observed cross-incompatibility among taxa support recognition of three North American species in *Nuttallanthus*. The extent and apportioning of

populational genetic variation within these taxa, uniformly low numbers of heterozygotes, and high degree of association between genetic distance among conspecific populations and geographic location are concordant with a mixed mating system characterized by autogamy and facultative xenogamy.

INTRODUCTION

The genus *Nuttallanthus* D. A. Sutton was established and segregated from *Linaria* Miller in 1988 and currently includes one South American species (*N. subandinus* (Diels) D. A. Sutton) and three species of native North American annual or biennial herbs: *N. canadensis* (L.) D. A. Sutton, *N. floridanus* (Chapman) D. A. Sutton and *N. texanus* (Scheele) D. A. Sutton (Sutton 1988; USDA/NRCS 2002). These plants possess heteromorphic stems and produce showy personate, bilabiate flowers with anterior nectar-storing spurs or pouches that attract a variety of Lepidopteran and Hemipteran visitors. Generic segregation as well as specific and varietal delimitation in this group of plants have been made primarily on the basis of variation in corolla morphology, spur length, and seed coat surface ornamentation. While the variation in floral morphology among taxa has been attributed to adaptation to different categories of pollinating insects, with *N. canadensis* and *N. texanus* attracting butterflies and *N. floridanus* being pollinated by flies (Pennell 1935), no systematic study of the mating system or pollination ecology of these species has been reported. These species commonly grow in dry sandy soils of dunes and open coniferous woodlands and as weeds of fields and other heavily disturbed areas. *Nuttallanthus canadensis* and *N. texanus* possess the greatest geographical ranges of any species among New World Antirrhineae (Elisens 1985). *Nuttallanthus canadensis* is native throughout much of temperate North America and is naturalized in South America and in Europe, where it has been cultivated as an ornamental for its showy, fragrant flowers (Tutin et al. 1972). *Nuttallanthus texanus* is native to the southern United States and Mexico, may be native to temperate South America, and is naturalized in other temperate regions (Sutton 1988).

Nuttallanthus floridanus is more narrowly distributed and occurs in the Atlantic and Gulf coastal plain of a few states in the southeastern U.S.A. Species ranges overlap, and all three taxa occur occasionally in dense, mixed populations.

The type species of the genus (*N. canadensis*) was described originally by Linnaeus (1753) as *Antirrhinum canadense* L. All native North American members of tribe Antirrhineae with spurred corollas were quickly reassigned to the genus *Linaria* by Miller (1754; 1768) and *L. canadensis* (L.) Dumort. was recognized in 1802 (Dumont de Courset 1802). *Linaria texana* Scheele (1848) and *L. floridana* Chapman (1860) were originally described as distinct species, but Pennell (1920, 1922) recognized only *L. canadensis* and *L. floridana* in his North American *Linaria* section *Leptoplectron*. *Linaria canadensis* was composed of two varieties: *L. canadensis* and *L. canadensis* var. *texana* (Scheele) Pennell that exhibited a high degree of intergradation in flower size. Munz (1926) concurred with Pennell's (1920) delimitation of *L. canadensis* and suggested that the intergradation in floral features between the two taxa was "quite complete" and that they could be distinguished reliably only on the basis of seed coat morphology. In 1935, Pennell reconsidered his earlier opinion and recognized *L. canadensis* and *L. texana* as distinct species, stating that the reported intergradation in flower size and seed structure could be attributed to ecological factors and hybridization among species. Several subsequent treatments relegated *L. texana* to varietal rank as *L. canadensis* var. *texana* (Scheele) Pennell (Rothmaler 1954; Cronquist et al. 1984, Hitchcock et al. 1998). In addition, plants possessing cleistogamous flowers and white corollas have been accorded infraspecific rank as *L. canadensis* forma *cleistogama* Fernald and *L. canadensis* forma *albina* Fernald, respectively (Fernald 1936; Fernald

1943). In a recent treatment of tribe Antirrhineae, Sutton (1988) recognized three native North American species in *Nuttallanthus* distinct from the Eurasian species of *Linaria*. Specific delimitations were made primarily on the basis of differences in seed coat structure, pedicel length, and corolla size. The extent of morphological variation within species and potential interspecific hybridization among species were not addressed (Sutton 1988).

Although species boundaries and evolutionary relationships are unresolved among New World toadflaxes, previous morphological studies supported hypotheses that the complex forms a monophyletic group distinct from the 150 Eurasian species of *Linaria* and from other New World members of tribe Antirrhineae. The size, surface morphology, and testal anatomy of the seeds of *Nuttallanthus* species are unique among New World Antirrhineae (Elisens et al. 1983; Elisens 1985), as is the floral structure (Rothmaler 1943), chromosome base number of $x = 6$, and pollen morphotype (Elisens 1986). In addition, *N. canadensis* possesses tubular nuclear inclusions in leaf mesophyll cells that are otherwise unique to *Linaria* among genera in Tribe Antirrhineae (Bigazzi 1989; Bigazzi 1993). Although pollen size and exomorphology of *Nuttallanthus* is similar to that of examined species of *Linaria* (Elisens 1986), the floral structure and seed coat morphology and anatomy of *Nuttallanthus* suggested that the New World species are taxonomically and phylogenetically distinct from Old World *Linaria* (Elisens et al. 1983; Elisens 1985; Sutton 1988).

This study employed starch gel electrophoresis of soluble enzymes and hand-pollination experiments to document the genetic variation and reproductive biology of the North American species of *Nuttallanthus*. The principal goals of this investigation were

to determine the amount of genetic differentiation within and among species in the genus, to test hypotheses of species boundaries and relationships, and to document the types of mating systems and the degree of reproductive and genetic isolation exhibited by these species.

MATERIALS AND METHODS

Isozyme analysis. A total of 649 individuals from 50 populations representing 3 species of *Nuttallanthus* were examined for electrophoretic variation: 325 individuals from 22 populations of *N. canadensis*, 110 individuals from 8 populations of *N. floridanus*, and 214 individuals from 20 populations of *N. texanus* (Table 1; Figure 1). Voucher specimens of the populations sampled were deposited at the Bebb Herbarium of the University of Oklahoma (OKL). Individual plants were collected by walking a transect along the greatest dimension of a population and collecting entire plants at intervals selected to attain a sample size appropriate for the size of that population; a maximum of 20 percent of any given population was sampled, although this limited the sample size obtainable from small populations. Individuals of populations 42 and 43 consisted of plants grown from field-collected seed. Plants were bagged individually and placed in plastic containers on ice for transport to the lab, where they were refrigerated at 4°C prior to protein extraction. Tissues from young, actively-growing leaves served as the enzyme source.

Fresh leaf material was ground in an extracting buffer consisting of 0.1 M tris-HCL pH 7.5, 1 mM EDTA (tetrasodium salt), 10 mM $MgCL_2$, 10 mM KCl, 14 mM beta-mercaptoethanol and 20 mg/ml solid polyvinylpyrrolidone (following Gottlieb 1981b).

Leaf extracts were centrifuged and the supernatant was absorbed onto wicks of Whatman 17 MM chromatography paper, which were then stored in 1.5 mL microcentrifuge tubes at -70°C for approximately 1 hour. Samples were electrophoresed on 11% starch gels using two buffer systems (Soltis et al. 1983) to resolve 15 loci for 10 enzyme systems: aspartate aminotransferase (AAT), alcohol dehydrogenase (ADH), α-glycerophosphate dehydrogenase (GPD), isocitrate dehydrogenase (IDH), malate dehydrogenase (MDH), 6-phosphogluconate dehydrogenase (6PGD), phosphoglucoisomerase (PGI), phosphoglucomutase (PGM), superoxide dismutase (SOD) and triosephosphate isomerase (TPI). System I consisted of an electrode buffer of 0.18 M Tris and 0.004 M EDTA titrated to pH 8.6 with boric acid and a gel buffer prepared from a 1:3 aqueous dilution of the electrode buffer; System I was used to resolve AAT, ADH, GPD, PGI, PGM, SOD and TPI. The electrode buffer of System II was prepared from 0.065 M L-histidine free base titrated to pH 6.5 with citric acid monohydrate and a gel buffer was obtained from a 1:3 aqueous dilution of the electrode buffer; System II was used to resolve IDH, MDH and 6PGD. Agarose-overlay and staining procedures used to detect enzyme activity followed the protocols of Soltis et al. (1983). The genetic bases of the enzyme banding patterns observed were inferred from their concordance with published data regarding the basic number of loci expected in the absence of gene duplication and patterns of enzyme expression (Crawford 1990; Gottlieb 1982). The documented number of independent banding regions and the patterns of banding within those regions were consistent with the known substructure and compartmentalization of the resolved enzymes. Where more than one locus and allele were observed, loci were numbered and alleles were lettered beginning with the most anodal (fastest-migrating) form.

Allele and genotype frequencies were determined for each population and species. The percentage of polymorphic loci, mean number of alleles per locus, mean number of alleles per polymorphic locus, mean observed heterozygosity, Nei's (1987) gene diversity, and the effective number of alleles per locus were calculated manually for each population and were averaged across all populations of each species. In addition, species-level statistics were computed by treating all sampled individuals within each species as members of a single population (following Hamrick et al. 1990); thus, the average genetic diversity of populations could be compared with the degree of genetic diversity found within each species. Spearman's rank-order correlation analysis was performed with SPSS for Windows (ver. 11.5.0; SPSS, Inc. 2002) to examine the association of population size with the genetic diversity of populations.

The total genetic diversity (H_T) within each species was partitioned into within-population (H_S) and among-population (D_{ST}) components (Nei 1973); the proportion of genetic diversity among populations (G_{ST}) and Wright's (1951) estimate of gene flow (N_m) were calculated for each species. To further assess the level of genetic differentiation among populations of each species of *Nuttallanthus*, Wright's F statistics (F_{IS}, F_{IT} and F_{ST}) were computed for each polymorphic locus and were averaged across loci within each species. Wright's fixation indices (F) were calculated for each locus and population of the three species of *Nuttallanthus*; the statistical significance of observed deviations of heterozygote proportions from Hardy-Weinberg expectations was assessed using a Chi square analysis (Wright 1965, 1978; Nei 1977). Nei's (1972) genetic distance and genetic identity values were computed for all pair-wise combinations of populations and species. Roger's distance as modified by Wright (1978) and Cavalli-

Sforza and Edwards (1967) chord distance coefficients were calculated and were used to produce dendrograms using the Distance Wagner procedure (Farris 1972); cophenetic correlations were calculated for each of the dendrograms. BIOSYS-1 (Swofford and Selander 1989) and GENESTAT2 (Whitkus 1988) provided computational software. NTSYSpc (ver. 2.11c; Rohlf 2002) was used to calculate Nei's (1972) genetic identity and distance, Roger's distance as modified by Wright (1978) and Cavalli-Sforza and Edwards (1967) chord distance for all pairwise comparisons among populations and species. Dendrograms were generated using these coefficients with the unweighted pair-group method using arithmetical averages (UPGMA) as discussed in Sneath et al. 1973; cophenetic correlations were calculated for each of the dendrograms. NTSYSpc was also used to conduct nonmetric-multidimensional scaling (MDS - Kruskal 1964) of Nei's (1972) genetic identities among populations. Species-level genetic diversity statistics and genetic distances and identities were calculated using all loci; only polymorphic loci were included in calculations of genetic diversity within and among populations, because statistics excluding monomorphic loci better represent the partitioning of genetic variation within and among populations (Berg and Hamrick 1997).

To assess the degree of association of the genetic distance between populations within each species and the geographic location of those populations, Spearman's rank-order correlation analysis was perfomed with SPSS for Windows on matrices of Nei's (1972) genetic distances and of linear distances (in kilometers) between populations.

Crossing studies. A minimum of five individuals was propagated from seed collected from each of 25 populations representing three species of *Nuttallanthus*. Due to the erratic germination of untreated seeds, seeds were soaked in a solution of gibberellic

acid (0.1g / L) for 10 minutes, washed with distilled water and germinated at room temperature on water-saturated filter paper in parafilm-sealed Petri dishes. Seedlings were transplanted to a well-drained soil-based potting soil mix and cultivated under pollinator-free conditions in a Conviron CMP2023 growth chamber (13.3 hrs of light at 28°C; nights at 16°C). The crossing program involved tests for autogamy (observation of untreated cleistogamous and chasmogamous flowers), apomixis (emasculation of flowers followed by no hand pollination), self-compatibility (emasculation followed by geitonogamous hand pollination) and cross-compatibility (emasculation followed by intra- and interspecific hand pollination). Buds of manipulated flowers were emasculated with needle-point forceps prior to anthesis, because the anthers of cleistogamous and chasmogamous flowers commonly dehisced prior to the opening of the perianth. Hand pollinations were accomplished one day following emasculation; forceps sterilized in 95% ethanol were used to transfer recently dehisced anther sacs to the receptive stigmas of emasculated flowers. Flowers used in reproductive experiments were not bagged. Due to the small size of the pre-anthesis floral structures, all floral manipulations were performed utilizing an Olympus dissecting microscope at 12.5X.

Two hundred forty hand pollinations representing all possible directional interspecific crosses were made; a minimum of 30 and a maximum of 50 hand pollinations were made for each directional interspecific combination. In addition, 75 or 80 intraspecific hand pollinations were performed among populations of each species; 100 hand pollinations using self pollen were performed for each species.

Seed production and germination rate were recorded for 100 capsules produced by both cleistogamous and chasmogamous flowers of each species and for all of the

manipulated flowers that produced mature capsules and seeds. Seeds were counted under a Wild M5 stereo microscope at 12X. 25 seeds from each capsule were soaked in a solution of gibberellic acid (0.1g / L) for 10 minutes, washed with distilled water and germinated at room temperature on water-saturated filter paper in parafilm-sealed Petri dishes.

Univariate statistical analyses were performed with SPSS for Windows (ver. 11.5.0; SPSS, Inc. 2002). Descriptive statistics of seed production and germination rate were calculated for each species; normality tests were performed for each treatment category. To compare treatment categories among populations and species, one-way analysis of variance was employed; post-hoc testing was performed using Fisher's least significant difference test.

RESULTS

Isozyme analysis. Fifteen loci coding for 10 enzymes were scored from populations of three species of *Nuttallanthus*: three for MDH, two for AAT, PGI and TPI, and one for ADH, GPD, IDH, 6PGD, PGM and SOD (Appendix 1); MDH-1 and MDH-2 overlapped so extensively that the loci and alleles could not be reliably distinguished; three banding patterns were observed, so MDH-1 and MDH-2 were treated collectively as a single polymorphic locus with patterns A, B and C corresponding to alleles (following Small et al. 1999). Additional isozymes were detected for AAT, ADH, IDH, 6PGD and PGM but were not scored due to low or inconsistent levels of activity or poor resolution. Additional enzyme systems detected included ME (using buffer system I); ALD, GA3PD, ME, MNR, and SDH were detected on buffer system II; these enzymes

were not scored due to low or inconsistent levels of activity or poor resolution. The number of isozymes detected was typical for diploid plant species, with the exception of MDH, which generally has three loci, and PGM, which normally has two (Gottlieb 1982; Weeden and Wendel 1989); no differences in isozyme number among species of *Nuttallanthus* was observed. The number of allelomorphs observed at polymorphic loci ranged from two (AAT-1, MDH-4, SOD) to five (AAT-2). One isozyme (MDH-3) was invariant in all examined individuals. Most of the populations of *Nuttallanthus texanus* examined in this study were composed of individuals possessing one of two principal allozyme profiles; individuals possessing different profiles were also distinguishable on the basis of seed coat morphology (Crawford 2003). One population in central Oklahoma (population 58) was composed of individuals belonging to both of these major groups of *N. texanus*; that population was divided into two sub-populations (58a and 58b) for analysis. Genotype data for populations are presented in Appendix 2; summary allele frequencies for three species of *Nuttallanthus* at 14 polymorphic loci are presented in Appendix 3.

Mean values for Nei's (1972) genetic identity coefficients (I) for pairwise comparisons of 50 populations within and among *Nuttallanthus* species are presented in Table 2. Average I values within species varied from 0.819 (*N. texanus*) to 0.936 (*N. canadensis*). Mean genetic identity values were considerably lower between species than within species, with interspecific I values ranging from 0.516 (*N. canadensis* x *N. floridanus*) to 0.623 (*N. canadensis* x *N. texanus*). Comparable patterns of genetic similarity were observed regardless of the coefficient employed (including Roger's distance as modified by Wright (1978) and Cavalli-Sforza and Edwards (1967) chord

distance). The average of all pairwise comparisons among *Nuttallanthus* species (I = 0.580) is considerably lower than the average identity value (I = 0.670) for plant congeners reported by Gottlieb (1981a).

The observed pattern of genetic similarities among species of *Nuttallanthus* results from both qualitative and quantitative allelic differences between those species. Many populations were fixed for a single allele at a majority of loci. The three species shared the same highest-frequency allele at 6 of 14 polymorphic loci. Of the 46 alleles observed among 14 polymorphic loci, 19 were unique to one of the three species; 3 "marker" alleles were either fixed or were present in high frequencies in all populations of a single species (6PGD-2c and TPI-1d in *N. canadensis*, and 6PGD-2b in *N. floridanus*). *Nuttallanthus floridanus* and *N. texanus* had the greatest number of alleles in common (20 of the 31 alleles detected in *N. texanus* were shared with *N. floridanus*); *N. texanus* shared an equal number of fixed or high-frequency alleles (9 of 24) with *N. canadensis* and *N. floridanus*, whereas *N. canadensis* and *N. floridanus* shared 6 fixed or high-frequency alleles.

Population- and species-level genetic diversity estimates based on allele frequencies are provided in Table 3. Within populations, the mean number of alleles per locus (A) ranged from 1.00 to 1.64; the mean number of alleles per polymorphic locus (A_p) varied from 2.0 to 3.0; and the percentage of polymorphic loci (P) ranged from 0.0 to 57.1. The average proportion of heterozygous loci per individual (H_o) ranged from 0.0 to 0.054; the average proportion of heterozygous loci per individual expected for populations in Hardy-Weinburg equilibrium (H_e) ranged from 0.0 to 0.283. Populations of *N. canadensis* exhibited the highest mean values of A (1.25), P (23.7) and H_e (0.076),

whereas *N. floridanus* populations showed the lowest levels of genetic variation among the three species. Spearman's rank-order correlation analyses indicated positive and statistically-significant associations between population (sample) size in *Nuttallanthus* and *A* ($r_s = 0.50$, $p < 0.001$), *P* ($r_s = 0.52$, $p < 0.001$), H_o ($r_s = 0.33$, $p = 0.019$), and H_e ($r_s = 0.49$, $p < 0.001$).

Estimates of genetic variation within populations of *Nuttallanthus* species were relatively low in comparison to values reported for other species of Scrophulariaceae (Elisens et al. 1988) and were similar to those reported for populations of selfing species and endemic species with narrow geographic ranges (Hamrick 1989; Hamrick et al. 1990). The effective mean number of alleles per locus (A_e) within populations varied from 1.00 to 1.39. A_e is equal to the actual number of alleles only when all alleles exist in equal frequency and provides a measure of allelic evenness; this value was lower than the mean number of alleles per locus (*A*) observed in all polymorphic populations, suggesting that a portion of the allelic diversity within populations was present in the form of low-frequency alleles (Nei 1987).

Genetic diversity within the three species of *Nuttallanthus* was considerably higher than that observed within populations of those species, with *N. texanus* showing values of *A* (2.21), *P* (71.4) and H_e (0.234) equal to or higher than those of *N. canadensis*; *N. floridanus* exhibited the lowest levels of genetic variation among the three species (Table 3). The total genetic diversity within each species (H_T), mean genetic diversity within populations (H_S) and among populations (D_{ST}), the proportion of genetic diversity among populations (G_{ST}) and Wright's (1951) estimate of gene flow (N_m) are presented in Table 4. Total gene diversity (H_T) ranges from 0.130 in *Nuttallanthus floridanus* to

0.242 in *N. texanus*. The low average values for H_S, ranging from 0.045 in *N. floridanus* to 0.079 in *N. canadensis*, demonstrated that little of the genetic variability within each species is present within individual populations. The relatively high average values for G_{ST}, varying from 0.420 in *N. canadensis* to 0.688 in *N. texanus*, indicated that considerable genetic differentiation exists among the populations of each species; these values were higher than the averages reported for annuals ($G_{ST} = 0.357$), species with regional distributions ($G_{ST} = 0.216$), and species of temperate regions ($G_{ST} = 0.246$); they were comparable to those reported for selfing species ($G_{ST} = 0.510$) (Hamrick et al. 1990). Estimates of gene flow ($N_m = (1 - G_{ST}) / 4G_{ST}$) ranged from 0.113 in *Nuttallanthus texanus* to 0.345 in *N. canadensis*; N_m estimates of less than 1.0 suggest relatively little gene flow among populations (Slatkin et al. 1989).

The observed and expected (for randomly outcrossing species) frequencies of heterozygous loci in the three species of *Nuttallanthus* are presented in Table 5. Observed heterozygosity was lower than expected heterozygosity for all polymorphic loci; the ratios of observed to expected heterozygosities were consistent with a selfing breeding system in all three species.

Wright's F statistics (F_{IS}, F_{IT} and F_{ST}) for polymorphic loci within each species of *Nuttallanthus* are provided in Table 6. The average amount of genetic variation distributed among populations (F_{ST}) ranged from 0.430 in *Nuttallanthus canadensis* to 0.694 in *N. texanus*. For all three species, the average total inbreeding coefficient (F_{IT}) appeared to be more greatly influenced by non-random mating within populations (F_{IS}) than by differences in allele frequencies among populations (F_{ST}). Of the 144 single-locus fixation indices tested, 138 (96 %) were both positive and significantly different

from Hardy-Weinberg expectations ($p < 0.05$), indicating a deficiency of heterozygotes at polymorphic loci in these species; an excess of heterozygotes was observed at a single locus (SOD) in a population of *Nuttallanthus canadensis*, but this excess was not significant (Appendix 4).

Dendrograms summarizing the genetic similarities among populations and species of *Nuttallanthus* were produced using the Distance Wagner procedure (Farris 1972) in combination with Roger's distance as modified by Wright (1978) and Cavalli-Sforza and Edwards (1967) chord distance coefficients, as well as with UPGMA (Sneath et al. 1973) in combination with Nei's (1972) genetic identity and distance. All of the dendrograms produced using the cited distance coefficients and clustering procedures were similar. Nei's (1972) genetic identity value resulted in the highest cophenetic correlation of the dendrograms produced (0.920); consequently, that dendrogram is reproduced here (Figure 2). The dendrogram clearly illustrates both geographic and taxonomic coherence; conspecific populations cluster together and, within individual species, populations tend to cluster with others from the same geographic region. Two discrete clusters of populations of *Nuttallanthus texanus* are apparent; one cluster is predominately composed of populations from the southeastern United States, California, Oklahoma and central Texas, whereas populations from Arkansas, Louisiana, eastern Texas and Oklahoma comprise the second cluster.

Nei's (1972) genetic identities among populations of *Nuttallanthus* species were subjected to nonmetric-multidimensional scaling (MDS) analysis; genetic similarity among populations was plotted in three dimensions (Figure 3). Final stress (a measure of the goodness of fit between the pairwise similarity coefficients in the original identity

matrix and those in the diagram produced by MDS) is 0.122, or "good to fair" (Kruskal 1964). The plot illustrates the pronounced genetic differentiation among the three species of *Nuttallanthus* and distinguishes the two principal groups of *N. texanus*.

Spearman's rank-order correlation analysis was performed with SPSS for Windows on matrices of Nei's (1972) genetic distances and of linear distances (in kilometers) between populations. These analyses indicated positive and statistically-significant associations between genetic distance and linear distance between populations for each species: *Nuttallanthus canadensis* ($r_s = 0.382$, $p < 0.001$), *N. floridanus* ($r_s = 0.635$, $p < 0.001$) and *N. texanus* ($r_s = 0.380$, $p < 0.001$).

Crossing studies. The results of seed production in three species of *Nuttallanthus* following controlled pollinations are presented in Table 7. Individuals of the three species of *Nuttallanthus* examined in this study were autogamous and commonly produced cleistogamous flowers both early and late in the life cycle; in these flowers, the limbs of the undeveloped corolla securely enclosed the male and female reproductive organs and self-fertilization occurred in the flower bud. The capsules produced by cleistogamous flowers were smaller and produced significantly fewer seeds than did those of chasmogamous flowers. Pollen appeared to be required for successful fruit and seed development, because no emasculated, unpollinated flower produced mature capsules and seeds. Within each of the three species of *Nuttallanthus*, no significant difference in seed production was observed between individuals pollinated with self pollen (geitonogamous hand pollination) or intraspecific cross pollen. Capsules produced through artificial hand pollination did yield significantly fewer seeds than did those from unmanipulated cleistogamous or chasmogamous flowers, which may have resulted from

limited pollen transfer between hand-pollinated flowers. The species of *Nuttallanthus* examined in this study appeared to be cross-incompatible. Of the 240 hand pollinations representing all possible directional interspecific crosses, none resulted in the successful development of capsules and seeds. While individuals of the two principal groups of *N. texanus* were readily distinguishable isozymically and morphologically, they appeared to be cross-compatible; no significant difference in seed production per fruit or seed germination rate was observed between the 35 hand pollinations made among individuals of the two principal groups of *N. texanus* and the 45 hand pollinations made among individuals within each group.

No statistically significant difference in seed production within any treatment category was observed between individuals of *Nuttallanthus canadensis* and *N. texanus*. Individuals of *N. floridanus* produced significantly fewer seeds per capsule for all treatments (other than emasculated, unpollinated flowers) compared to its congeners. No significant difference in the germination rate of seeds was observed among any species or treatment category.

DISCUSSION

Allozyme variation and life history characteristics: *Nuttallanthus canadensis*, *N. texanus* and *N. floridanus* exhibited moderate levels of genetic variation, comparable to that reported for other species with similar (regional to widespread) geographic ranges (Hamrick 1989; Hamrick et al 1990). Much of the genetic variance present in plant species has been interpreted in terms of population size and geographic range: species with small populations and restricted or narrowly-endemic distributions often possess

considerably less genetic diversity than do species with large population sizes and widespread ranges. Geographic range is the single best predictor of species-level genetic diversity (Hamrick et al 1990). This pattern is evident in *Nuttallanthus*, where the more narrowly distributed *N. floridanus* exhibited lower levels of genetic variation than did its widespread congeners (Table 3).

The level of genetic variation found within individual populations of the three *Nuttallanthus* species was considerably lower than that observed at the species level. Previous investigations and reviews have indicated that species-level genetic diversity is significantly and positively associated with population-level genetic diversity in plants, and that little (approximately 22%) of the genetic variation in a species occurs among populations of that species (Gottlieb 1981a; Hamrick et al. 1990). However, species-level and population-level genetic diversity are not strongly associated in species of North American *Nuttallanthus*. Although the percentage of polymorphic loci observed in *Nuttallanthus* species (ranging from 64.3% to 71.4%) was well above the average reported for plant species (50.5%), most populations of *Nuttallanthus* were fixed for a single allele at a majority of loci (Table 3; Appendix 1). In addition, *Nuttallanthus* species had from 42% (*N. canadensis*) to 69% (*N. texanus*) of their genetic variation distributed among populations, whereas relatively little diversity existed within populations of these species (Table 4; Table 6). A number of life history characteristics have been shown to have profound influence upon the extent and organization of genetic variation within plant species (Gottlieb 1981a; Crawford 1990). Low chromosome numbers (n = 6 in species of *Nuttallanthus*; Crawford 2003) often are associated with low levels of infraspecific genetic variation (Hamrick 1989), and the maintenance of high

levels of genetic differentiation among populations has been associated with physical distance between populations (Wright 1969; Gibson et al. 1991); correlation analyses indicated positive and statistically-significant associations between genetic distance and the linear distance between conspecific populations of *Nuttallanthus*. In addition, demographic, geographical (spacial substructuring within and among populations) and reproductive characteristics appear to affect the type and amount of genetic variation observed among North American species of *Nuttallanthus* and the development and maintenance of genetic differentiation among populations of these species.

Nuttallanthus canadensis, *N. texanus* and *N. floridanus* are herbaceous annuals (occasionally biennials) that occupy seral and disturbed habitats and that produce large numbers of seeds per fruit and per individual (have high fecundity). Dramatic annual changes in population size are observed often in these species (pers. observ.). In addition, field observations and the early successional status of these adventive species suggest that populations may be founded by a limited number of individuals. The combination of founding effects and the occasional reduction of populations to a small number of individuals could act to depress genetic variation within populations and to promote differentiation among populations of these taxa. Population (sample) size was significantly correlated with several commonly cited measures of genetic diversity in species of *Nuttallanthus*, including the percentage of polymorphic loci, mean number of alleles per locus, and the observed and expected levels of heterozygosity. These results indicated that allozyme variation within these taxa is affected by population size and suggest that the amount and type of genetic variation in these species has been affected by genetic drift (Nei et al. 1975).

The high genetic variability values observed in a small population of *N. texanus* (population 59), in comparison to the low variability estimates of other comparably-sized populations of this species (Table 3), as well as the notably-high level of populational genetic differentiation in *N. texanus* in comparison to its congeners (Table 4) suggest that spacial structure within and among populations may also influence populational genetic diversity in this species. The majority of the populations sampled in this study appeared to be structurally-homogeneous; however, population 59 consisted of two well-defined subpopulations: small groups of individuals were found growing in 2 "islands" of soil in shallow depressions in a granitic outcrop. While these subpopulations were separated by a distance of no more than 25 meters, individuals from each subpopulation were fixed for different alleles at 7 of 14 polymorphic loci (Appendix 2), dramatically increasing the estimates of genetic diversity in this population. The high level of genetic differentiation observed among populations of *N. texanus* relative to its congeners may be attributed to the presence in this species of two largely allopatric and genetically-distinctive allelic groups of populations: one group (Group 1) composed of populations from the southeastern United States, California, Oklahoma and central Texas, and a second (Group 2) comprised of populations from Arkansas, Louisiana, eastern Texas and Oklahoma. While these groups were fixed for the same allele at 4 of 14 polymorphic loci, quantitative allelic differences were observed at several loci (AAT-2, GPD, PGM-1, TPI-2), at which populations of the two groups exhibited different fixed or high-frequency alleles; nine of the 31 alleles observed in Group 1 were not found in Group 2.

Other life history characteristics found in *Nuttallanthus* species associated with the promotion of populational genetic differentiation include traits that act to inhibit gene

flow among individuals and populations, such as seeds with limited dispersal capacity and an autogamous mating system. Seeds of *Nuttallanthus* are very small (approximately 300 microns in length) and are lacking in any suface ornamentation commonly associated with wind dispersal; these seeds appear to be gravity-dispersed. Indirect estimates of gene flow (N_m; Table 4) in species of *Nuttallanthus* based on allozyme data indicate limited gene flow among populations of these taxa (Wright 1951; Slatkin et al. 1989); these estimates are comparable to those reported for selfing species with small, gravity-dispersed seeds (Hamrick 1989). Selfing species generally possess lower absolute levels of genetic variation than do species with mixed or outcrossing mating systems, and a greater proportion of the variation within selfing species is apportioned among individual populations; conversely, outcrossing taxa tend to sequester higher levels of genetic variation within - rather than among - populations (Brown 1979; Loveless et al 1984). The extent and apportionment of genetic variation observed in North American *Nuttallanthus* was congruent with the predominately-selfing mating system of these taxa. Individuals of these species commonly produce cleistogamous (obligately-selfing) flowers; the anthers of chasmogamous flowers generally dehisced prior to the opening of the perianth, coating the immediately-adjacent and receptive stigmas with self-pollen and potentially inhibiting outcrossing. No significant difference in seed production was observed between individuals artificially pollinated with self pollen and those pollinated with intraspecific cross pollen, indicating that these species are completely self-compatible (Table 7). Field tests conducted in two Oklahoma populations of *N. texanus* (and involving five treatments: open pollination, pollinator exclusion, floral emasculation and pollinator exclusion, emasculation and open-pollination, and hand/self-pollination of

emasculated flowers) provided similar results. These tests indicated *N. texanus* is facultatively xenogamous; while many potential Lepidopteran and Hemipteran pollinators were observed visiting successive flowers among different plants, successful pollen transfer and seed set was rare (Crawford, unpublished data). The high level of selfing observed in these species was consistent with the deficiency of heterozygotes observed at all polymorphic loci (Table 5; Appendix 4).

Systematic implications: The observed patterns of allozyme divergence in North American *Nuttallanthus* are consistent with recognition of three distinct species with no infraspecific taxa: *N. canadensis*, *N. floridanus* and *N. texanus*, as proposed by Sutton (1988). Analyses of genetic divergence readily distinguished three principal groups in the genus. Qualitative and quantitative allelic differences among species were evident at many loci; 41% of the observed alleles were unique to one of the three species and 4 marker alleles were either fixed or present in high frequencies in all populations of a single species (two in *N. canadensis* and one each in *N. floridanus* and *N. texanus*). Pairwise genetic identity values between species (average of 0.580) were much lower than infraspecific identities (average I value of 0.888; Table 2). Although a wide range of infrageneric identity values has been documented in plant species, the observed interspecific genetic identity (I) values in *Nuttallanthus* were lower than those reported for most congeneric angiosperm species (Crawford 1983) and the mean value of 0.67 reported by Gottlieb (1981a). These results indicate that considerable allozyme divergence has occurred subsequent to the interruption of gene flow among these species.

The extent of genetic divergence among species is concordant with the observed cross-incompatibility among species; no artificial interspecific cross pollinations resulted

in the development of mature capsules and seeds and no individuals of purported hybrid origin were observed in natural populations. Although cross-compatibilities vary within populations of some plants, our observations of interspecific cross-incompatibility in *Nuttallanthus* supports hypotheses indicating a long period of isolation since speciation (Grant 1981; Raven 1977). Species of *Nuttallanthus* appear to have similar habitat requirements, are sympatric in a portion of their ranges (often occurring in dense, mixed populations of two or three species) and exhibit similar flowering phenologies; many potential Lepidopteran and Hemipteran pollinators were observed visiting successive flowers among plants of different species (pers. observ.). While cleistogamy and the early dehiscence of the anthers of chasmogamous flowers may act to limit interspecific cross-pollination, no other premating isolating mechanism was evident among these species. However, crossing studies indicated the presence of some pre- or post-zygotic barrier to hybridization, and no evidence of hybridization between species was apparent in the isozyme data.

The dendrograms summarizing the genetic similarities among populations of *Nuttallanthus* were robust with respect to different distance measures and clustering algorithms; all of the dendrograms delineated three principal groups of populations corresponding to the three currently-recognized North American species of *Nuttallanthus* (Figure 2). Similar results were obtained through nonmetric-multidimensional scaling analysis (MDS) of genetic identities among populations (Figure 3). While these results illustrated the considerable allozymic divergence among these species, they did not clearly resolve phylogenetic relationships among species of *Nuttallanthus*. These species did not appear to differ in isozyme number, and the broadly similar estimates of genetic

divergence among species renders inferences regarding their evolutionary relationships suspect. Recently derived plant species commonly exhibit a subset of the genetic variation present in the progenitor species, and little or no qualitative divergence at isozyme loci is evident between progenitor-derivative species pairs (Crawford 1990; Loveless et al. 1988). The extent of the genetic divergence among species of *Nuttallanthus* (low identity values and significant frequency differences among shared alleles) and the nature of that divergence (numerous unique and marker alleles in each species) provide no evidence of a recent derivation of these taxa.

While plants possessing cleistogamous flowers have been accorded infraspecific status as *L. canadensis* forma *cleistogama* Fernald (Fernald 1936), field observations and breeding system studies indicate that many (if not all) individuals of the three species of *Nuttallanthus* are capable of producing cleistogamous flowers both early and late in the life cycle; consequently, the recognition of infraspecific cleistogamous taxa in this genus is unjustified.

Infraspecific identity values in *N. texanus* (I = 0.819) were considerably lower than those found among populations of its congeners (I = 0.936 in *N. canadensis* and I = 0.909 in *N. floridanus*), demonstrating that considerable interpopulational genetic divergence has occurred in this species. Identity values within the two principal groups of *N. texanus* (I = 0.878 in Group 1 and I = 0.928 in Group 2) were comparable to those of other *Nuttallanthus* species, and the degree of genetic similarity between Groups 1 and 2 (I = 0.737) was intermediate between the average infraspecific (I = 0.888) and infrageneric (I = 0.580) values observed in *Nuttallanthus*. Populations of the two groups of *N. texanus* were largely allopatric (with the exception of population 58), a situation

observed in other infraspecific taxa exhibiting lowered genetic identities (Crawford 1989). Nine of the 31 alleles observed in Group 1 were absent in Group 2, which possessed no unique alleles; Group 2 contained a subset of the total genetic variation present in Group 1 (Appendix 2). These results suggest that gene exchange has been restricted between these groups of populations for a considerable period of time. Whereas allozyme data indicated a substantial degree of genetic divergence between these two groups, breeding system experiments illustrated no reduction in interfertility. No significant difference in the production or germination rate of seeds was observed between the artificial pollinations made between individuals of Groups 1 and 2 and the pollinations made among individuals within each group; however, the study provided no information regarding the post-germination viability and fertility of the hybrid offspring, so the potential for hybrid sterility or hybrid (F_2) breakdown may exist.

Biogeographic patterns: The extant center of taxonomic diversity of *Nuttallanthus* lies in the east Gulf Coastal Plain of Alabama and Florida where all three species are sympatric. This region harbors a large number of endemic species and genera, has been recognized as a center of plant endemism, and may have served as a refuge for many plant species during cycles of Pleistocene glaciation (Delcourt et al. 1981; Estill et al. 2001). While the flora of the Atlantic and Gulf coastal plain has been considered to be of geologically-recent origin (Thorne 1993), the large number of genera endemic to this region indicates that some floristic elements are of greater antiquity (Sorrie et al. 2001). Nei's (1987) stepwise mutation rate models (I_E and I_{EA}) based on genetic identities among species of *Nuttallanthus* provided estimates of the elapsed time since divergence of *N. canadensis* and *N. texanus* ranging from 2.75 to 4.0 million years

(my) BP; estimated divergence times for *N. texanus* and *N. floridanus* ranged from 3.0 to 4.5 my BP, and those for *N. canadensis* and *N. floridanus* ranged from 5.0 to 7.0 my BP. These models suggested that species of *Nuttallanthus* diverged in the late Tertiary (late Miocene and Pliocene). Although much of the Atlantic and Gulf coastal plain was inundated by advancing seas during portions of the Tertiary and Pleistocene, isolated areas of the coastal plain were suitable for plant habitation by the late Miocene (Walker et al. 1987). These fragmented areas of the coastal plain apparently served as refugia for a number of archaic plant taxa (Sorrie et al. 2001) and may have provided opportunities for speciation among geographically-isolated ancestral populations of *Nuttallanthus*.

In addition to the extensive genetic divergence between species of *Nuttallanthus*, individual taxa exhibited genetic substructuring at a regional level. Significant correlations were evident between genetic distance and the physical distance between conspecific populations of *Nuttallanthus*, and clustering and ordination analyses consistently grouped conspecific populations with others from the same geographic region. Many of these population clusters are located in geographic regions corresponding to recognized centers of plant endemism, where environmental conditions have fostered speciation or allowed refugial taxa to persist. UPGMA of Nei's (1972) genetic identities among populations (Figure 2) illustrated genetic divergence between three groups of *N. canadensis*: 1) populations located on the Delmarva peninsula and separated from southern and western conspecific populations by the Chesapeake Bay and the Potomac River; 2) populations found on the Mid-Atlantic Coastal Plain of Virginia, North Carolina and Georgia and the Outer Banks of North Carolina; and 3) populations located on the Southern Atlantic and Gulf coasts of the United States (and one population

from Massachusetts). The modern Chesapeake Bay corresponds to the geologic Salisbury embayment, which has been recognized as a major biogeographic barrier; many plant species found in the middle- and southern Atlantic and Gulf coastal plain do not occur to the north of this break (Sorrie et al. 2001). The Mid-Atlantic Coastal Plain and the Apalachicola region in the Panhandle of Florida are separated by several large river systems (including the Cape Fear and Savannah Rivers) which have influenced the distributional patterns of many plant taxa (Estill et al. 2001) and which have apparently formed partial barriers to gene flow among populations of *N. canadensis*. The clustering of the Massachusetts population (20) with those of the Southern Atlantic and Gulf coast populations conforms to no obvious biogeographic pattern; the location of population 20 may be due to anthropogenic dispersal of seeds. *N. canadensis* and *N. texanus* commonly occur in agricultural settings, including fallow fields and stands of alfalfa *(Medicago sativa)*; the seeds of *Nuttallanthus* are similar in size to those of alfalfa and may function as crop mimics. However, the pronounced spatial and genetic structuring of populations within *Nuttallanthus* suggests that such anthropogenic dispersal has been relatively rare.

Populations of *N. floridanus* also exhibited a pronounced geographic pattern in divergence at isozyme loci, with two groups of populations occurring on the Gulf coastal plain and separated by the Apalachicola River, and a single outlier located in central pensinsular Florida. The Apalachicola region of the Florida Panhandle and central peninsular Florida have been identified as centers of plant endemism, and the Apalachicola/Chattahoochee River system has constituted a barrier to migration and gene flow in a number of coastal plain taxa (Avise 1992; Avise et al. 1979; Sorrie et al. 2001).

Concordance between geographic isolation and genetic differentiation among populations is also apparent in *N. texanus*; UPGMA identified clusters of populations located in California, Oklahoma and Texas, and in the southeastern United States (Figure 2). The populations comprising group 2 of this species are largely allopatric from those of group 1 and are located in and near the former Mississippi Embayment.

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Table 1. Numbered collection data for 60 populations representing three species of *Nuttallanthus* examined for isozyme variation. Population codes are given in bold numbers; OTUs included in population level isozyme analyses are indicated by an asterisk (*). Numbers of individuals per OTU examined for isozyme variation are given in parentheses, followed by the collection numbers for those specimens.

Nuttallanthus canadensis

Alabama: Baldwin Co.: **1*** = 30.414N 87.598W, fallow cornfield 0.1 mi. NE of Highway 98 & S Michigan, Elberta, AL (n=16, *Crawford 358-373)*; **2** = 30.238N 87.882W, open pine woods N of Miller Memorial Cemetery, Miller Memorial Road and Highway 180 intersection (n=5, *Crawford 686-690)*. Mobile Co.: **3** = 30.433N 88.144W, grounds of Bellingrath Gardens Estate, near picnic grounds (n=10, *Crawford 324-333)*; **4** = 30.243N 88.078, E end of Dauphin island in seaside dunes (n=2, *Crawford 650-651)*.

Delaware: Sussex Co.: **5*** = 38.574N 75.056W, sand dunes in meadow W of Highway 1, 2.0 mi. N of Highway 1 & Highway 26 intersection (n=30, *Crawford 211-240)*.

Florida: Bay Co.: **6*** = 30.204N 85.847W, S of Beach Front Road, 3.0 mi. W of Highway Alt98 & Highway 392 intersection (n=25, *Crawford 380-399, 714-718)*; Calhoun Co.: **7** = 30.464N 85.045W, fallow cornfield 1.0 mi. N of Blountstown (n=8, *Crawford 853-860)*. Franklin Co.: **8*** = 29.833N 84.876W, meadow S of Highway 65, 8.6 mi. N of Highway 65 & Highway 98 intersection (n=19, *Crawford 406-415, 417-425)*; **9*** = 29.909N 84.394W, dunes on Lighthouse Point, W of Alligator Point village, W end of peninsula (n=13, *Crawford 449-461)*; **10** = 29.853N 84.664W, open pine woodland near Carrabelle, N of 3rd Street (n=3, *Crawford 808-810)*. Lafayette Co.: **11*** = 30.139N 83.290W, improved pasture 0.1 mi. N of Highway 27, 8.8 mi. W of Mayo (n=10, *Crawford 493-502)*. Wakulla Co.: **12*** = 30.136N 84.326W, meadow 0.1

Table 1 cont.

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mi. NE of Highway 98 & Spring Creek Highway intersection (n=10, *Crawford 817-826)*. Walton Co.: **13** = 30.309N 86.102W, shore of Eastern Lake, E of Seagrove Beach, near Highway 30A (n=5, *Crawford 705-709)*.

Georgia: Camden Co.: **14*** = 30.759N 81.658W, meadow above N bank of St. Mary's River, 0.1 mi. E of I95 (n=20, *Crawford 509-528)*. Candler Co.: **15*** = 32.355N 81.989W, meadow 0.3 mi. N of Interstate 16, 11.7 mi. SE of Interstate 16 & Highway 57 intersection (n=10, *Crawford 596-605)*. Glynn Co.: **16*** = 31.020N 81.435W, meadow near St. Andrew picnic area, S Riverside Drive, Jekyll Island (n=10, *Crawford 532-541)*. Liberty Co.: **17*** = 31.675N 81.414W, edge of Beltowne marsh 3.6 mi. S of Retreat (n=10, *Crawford 545-554)*.

Maryland: Caroline Co.: **18*** = 38.817N 75.748W, wet meadow S of Highway 404, 1.5 mi. E of Highway 404 & Highway 16 intersection (n=10, *Crawford 244-253)*. Worcester Co.: **19*** = 38.096N 75.499W, fallow cornfield E of Highway 113, 0.3 mi. S of mile 6 marker (n=17, *Crawford 186-202)*.

Massachusetts: Middlesex Co.: **20*** = 42.504N 71.265W, sandy roadside at edge of pine woodland, Concord Field Station, Bedford (n=15, *Crawford 901-915)*.

North Carolina: Currituck Co.: **21*** = 36.278N 75.915W, 0.5 mi. N of Highway 158 & Highway 3E intersection (n=10, *Crawford 118-127)*. Dare Co.: **22*** = 35.261N 75.579W, Hatteras Island, near intersection of Highway 12 & Paradise Lane (n=10, *Crawford 104-113)*. Duplin Co.: **23*** = 34.926N 77.652W, S of Highway 24, 1 mi. W of Duplin/Onslow county line (n=10, *Crawford 91-100)*. Hoke Co.: **24*** = 35.007N 79.305W, SW of intersection of Highway 211 & SR1202 (n=20, *Crawford 68-87)*.

Table 1 cont.

South Carolina: Beaufort Co.: **25*** = 32.377N 80.832W, meadow on W bank of Chechessee R, 0.1 mi. SE of Highway 170 bridge (n=10, *Crawford 573-582).*

Virginia: Accomack Co.: **26*** = 37.912N 75.356W, Chincoteague Island, S of Highway 175, near causeway to Assateague Island (n=20, *Crawford 160-179).* Northampton Co.: **27*** = 37.145N 75.967W, meadow 0.2 mi. E of Highway 13, 0.4 mi. S of Highway 13 & Latimer Siding Road intersection (n=20, *Crawford 134-153).* Orange Co.: **28*** = 38.261N 77.980W, near Highway 20 & Village Road intersection (n=10, *Crawford 257-266).*

Nuttallanthus floridanus

Alabama: Baldwin Co.: **29*** = 30.238N 87.882W, open pine woods N of Miller Memorial Cemetery, Miller Memorial Road and Highway 180 intersection (n=11, *Crawford 673-683).* Mobile Co.: **30*** = 30.243N 88.078, E end of Dauphin island in seaside dunes (n=11, *Crawford 657-667).*

Florida: Bay Co.: **31*** = 30.204N 85.847W, S of Beach Front Road, 3.0 mi. W of Highway Alt98 & Highway 392 intersection (n=25, *Crawford 723-747).* Franklin Co.: **32*** = 29.909N 84.394W, dunes on Lighthouse Point, W of Alligator Point village, W end of peninsula, (n=10, *Crawford 467-476);* **33*** = 29.723N 84.890W, 0.2 mi. S of Highway 98 & Highway 300 intersection (n=8, *Crawford 753-760);* **34*** = 29.724N 84.899W, sandy meadow SE of E end of Gorrie Bridge, E bank of Apalachicola River (n=20, *Crawford 772-791);* **35*** = 29.853N 84.664W, open pine woodland near Carrabelle, N of 3rd Street (n=10, *Crawford 797-806).* Putnam Co.: **36*** = 29.623N 81.912W, open pine woodland 0.1 mi. S of Highway 20, W of Interlachen (n=15, *Crawford 837-851).*

Table 1 cont.

Nuttallanthus texanus

Alabama: Baldwin Co.: **37*** = 30.414N 87.598W, fallow cornfield 0.1 mi. NE of Highway 98 & S Michigan, Elberta (n=14, *Crawford 339-352)*. Mobile Co.: **38*** = 30.243N 88.078, E end of Dauphin island in seaside dunes (n=10, *Crawford 639-648)*.

Arkansas: Conway Co.: **39*** = 35.171N 92.755W, ca. 0.1 mi. S of Interstate 40 mile marker 107 (n=10, *Crawford 52-61)*. Crawford Co.: **40*** = 35.528N 94.041W, ca. 0.25 mi. S of Interstate 40, 1.0 mi. W of Mulberry exit (n=10, *Crawford 39-48)*; Logan Co.: **41** = 35.300N 93.634W, Subiaco Academy grounds, Subiaco (n=5, *PT Crawford 270-274)* [Logan].

California: Monterrey Co.: **42*** = 36.511N 121.942W, grown from seed collected at Point Lobos State Reserve (n=5, *Crawford 920-924)*. Santa Barbara Co.: **43*** = 34.044N 119.718W, grown from seed collected at Pelican Bay, Santa Cruz Island (n=5, *Crawford 929-933)*.

Florida: Calhoun Co.: **45** = 30.464N 85.045W, fallow cornfield 1.0 mi. N of Blountstown (n=4, *Crawford 862-865)*. Franklin Co.: **46** = 29.833N 84.876W, 8.6 mi. N of Highway 65 & Highway 98 intersection (n=1, *Crawford 416)*; **47*** = 29.909N 84.394W, dunes on Lighthouse Point, W of Alligator Point village, W end of peninsula (n=13, *Crawford 431-443)*; **48** = 29.723N 84.890W, 0.2 mi. S of Highway 98 & Highway 300 intersection (n=5, *Crawford 762-766)*; **49** = 29.853N 84.664W, open pine woodland near Carrabelle, N of 3rd Street (n=1, *Crawford 811)*. **50*** = Wakulla Co.: 30.136N 84.326W, meadow 0.1 mi. NE of Highway 98 & Spring Creek Highway intersection (n=13, *Crawford 480-489, 829-831)*. Walton Co.: **51*** = 30.309N 86.102W, shore of Eastern Lake, E of Seagrove Beach (n=7, *Crawford 696-702)*.

Georgia: Candler Co.: **52*** = 32.355N 81.989W, meadow 11.7 mi. SE of Interstate 16 and Highway 57 intersection (n=5, *Crawford 587-591)*.

Table 1 cont.

Louisiana: St. Landry Parish: **53*** = 30.540N 92.028W, meadow 0.2 mi. E of Interstate 49 & Highway 190 intersection (n=10, *Crawford 296-305)*. St. Tammany Parish: **54*** = 30.298N 89.666W, meadow 0.3 mi. S of Interstate 12, high on W bank of Pearl River (n=12, *Crawford 309-320)*.

Oklahoma: Cleveland Co.: **56*** = 35.214N 97.328W, T9N R1W Sec 32 NW 1/4 (n=10, *Crawford 626-635)*. Garvin Co.: **57*** = 34.708N 97.543W, T3N R3W Sec 30 NE 1/4 (n=20, *Crawford 606-625)*; **58*** = 34.745N 97.548W, T3N R3W Sec 19 SE 1/4 (n=25, *Crawford 944-968)*. Johnston Co.: **59*** = 34.327N 96.770W, T3S R5E Sec 3 NE 1/4, "islands" of shallow soil in Tishomingo granite of Ten-acre Rock (n=10, *Crawford 888-897)*.

South Carolina: Beaufort Co.: **60*** = 32.377N 80.832W, meadow on W bank of Chechessee R, 0.1 mi. SE of Highway 170 bridge (n=10, *Crawford 559-568)*.

Texas: Harrison Co.: **61*** = 34.470N 94.595W, pasture 0.1 mi. N of Interstate 20, 0.8 mi. W of exit 604 (n=10, *Crawford 283-292)*. Smith Co.: **62*** = 32.469N 95.389W, improved pasture 0.25 mi. S of Interstate 20 near mile marker 557 (n=15, *Crawford 871-885)*.

Table 2. Mean values for Nei's (1972) Genetic Identity (I) coefficients for pairwise comparisons of 50 populations within and among three species of *Nuttallanthus*. Ranges are given in parentheses; numbers of sampled populations / individuals are given in brackets.

Species	*N. canadensis*	*N. floridanus*	*N. texanus*
N. canadensis [22 / 325]	0.936 (0.834 - 0.998)		
N. floridanus [8 / 110]	0.516 (0.408 - 0.655)	0.909 (0.817 - 1.000)	
N. texanus [20 / 214]	0.623 (0.449 - 0.765)	0.601 (0.429 - 0.770)	0.819 (0.604 - 1.000)

Table 3. Summary of allozyme variation for 14 putative loci within 22 populations of *Nuttallanthus canadensis,* 8 populations of *N. floridanus* and 20 populations of *N. texanus.* N = sample size, A = mean number of alleles per locus, A_p = mean number of alleles per polymorphic locus, P = % polymorphic loci, H_o = mean observed heterozygosity, H_e = mean expected heterozygosity, and A_e = effective number of alleles.

N. canadensis

Population	N	A	A_p	P	H_o	H_e	A_e
1	16	1.50	2.17	42.9	0.004	0.162	1.19
5	30	1.29	2.00	28.6	0.010	0.101	1.11
6	25	1.50	2.00	50.0	0.006	0.119	1.14
8	19	1.43	2.00	42.9	0.011	0.098	1.11
9	13	1.43	2.20	35.7	0.000	0.140	1.16
11	10	1.14	2.00	14.3	0.007	0.045	1.05
12	10	1.07	2.00	7.1	0.000	0.034	1.04
14	20	1.29	2.00	28.6	0.011	0.055	1.06
15	10	1.29	2.00	28.6	0.000	0.069	1.07
16	10	1.14	2.00	14.3	0.021	0.067	1.07
17	10	1.14	2.00	14.3	0.000	0.064	1.07

Table 3 cont.

Population	N	A	A_p	P	H_o	H_e	A_e
18	10	1.29	2.00	28.6	0.007	0.078	1.08
19	17	1.36	2.00	35.7	0.008	0.105	1.12
20	15	1.00	-	0.0	0.000	0.000	1.00
21	10	1.14	3.00	7.1	0.000	0.033	1.03
22	10	1.07	2.00	7.1	0.007	0.027	1.03
23	10	1.36	2.00	35.7	0.007	0.137	1.16
24	20	1.21	2.00	21.4	0.004	0.091	1.10
25	10	1.21	2.00	21.4	0.000	0.087	1.10
26	20	1.36	2.25	28.6	0.004	0.071	1.08
27	20	1.29	2.00	28.6	0.014	0.092	1.10
28	10	1.00	-	0.0	0.000	0.000	1.00
Mean	**14.8**	**1.25**	**2.08**	**23.7**	**0.006**	**0.076**	**1.09**
(SD)	**5.74**	**0.147**	**0.224**	**13.9**	**0.005**	**0.042**	**0.049**
Species level	**325**	**2.14**	**2.60**	**71.4**	**0.006**	**0.134**	**1.15**
(SD)	---------	**0.915**	**0.663**	---------	---------	**0.154**	---------

Table 3 cont.

N. floridanus

Population	N	A	A_p	P	H_o	H_e	A_e
29	11	1.00	-	0.0	0.000	0.000	1.00
30	11	1.00	-	0.0	0.000	0.000	1.00
31	25	1.21	2.00	21.4	0.000	0.089	1.10
32	10	1.29	2.00	28.6	0.000	0.079	1.09
33	8	1.29	2.33	21.4	0.000	0.069	1.07
34	20	1.14	2.00	14.3	0.000	0.020	1.02
35	10	1.07	2.00	7.1	0.000	0.034	1.04
36	15	1.21	2.00	21.4	0.005	0.053	1.06
Mean	**13.8**	**1.15**	**2.06**	**14.3**	**0.001**	**0.043**	**1.05**
(SD)	**5.52**	**0.111**	**0.123**	**10.1**	**0.002**	**0.033**	**0.036**
Species level	**110**	**1.86**	**2.33**	**64.3**	**0.001**	**0.119**	**1.14**
(SD)	---	**0.742**	**0.471**	---	---	**0.179**	---

Table 3 cont.

N. texanus

Population	N	A	A_p	P	H_o	H_e	A_e
37	14	1.50	2.17	42.9	0.000	0.152	1.18
38	10	1.14	2.00	14.3	0.000	0.026	1.03
39	10	1.00	-	0.0	0.000	0.000	1.00
40	10	1.07	2.00	7.1	0.000	0.013	1.01
42	5	1.00	-	0.0	0.000	0.000	1.00
43	5	1.00	-	0.0	0.000	0.000	1.00
47	13	1.21	2.00	21.4	0.000	0.079	1.09
50	13	1.29	2.00	28.6	0.011	0.115	1.13
51	7	1.14	2.00	14.3	0.054	0.050	1.05
52	5	1.00	-	0.0	0.000	0.000	1.00
53	10	1.21	2.00	21.4	0.007	0.084	1.09
54	12	1.36	2.00	35.7	0.012	0.124	1.14
56	10	1.14	2.00	14.3	0.000	0.036	1.04
57	20	1.29	2.00	28.6	0.007	0.104	1.12

Table 3 cont.

Population	N	A	A_p	P	H_o	H_e	A_e
58a	13	1.57	2.14	50.0	0.005	0.194	1.24
58b	12	1.14	2.00	14.3	0.012	0.055	1.06
59	10	1.64	2.13	57.1	0.000	0.283	1.39
60	10	1.14	2.00	14.3	0.000	0.026	1.03
61	10	1.07	2.00	7.1	0.000	0.013	1.01
62	15	1.21	2.00	21.4	0.000	0.104	1.12
Mean	**10.7**	**1.21**	**2.03**	**19.6**	**0.005**	**0.073**	**1.09**
(SD)	**3.54**	**0.183**	**0.058**	**16.3**	**0.012**	**0.073**	**0.096**
Species level	**214**	**2.21**	**2.70**	**71.4**	**0.005**	**0.234**	**1.31**
(SD)	---	**0.860**	**0.458**	---	---	**0.223**	---

Table 4. Genetic diversity statistics for *Nuttallanthus canadensis, N. floridanus* and *N. texanus*. H_T = total gene diversity, H_S = gene diversity within populations, D_{ST} = gene diversity among populations, G_{ST} = the proportion of gene diversity apportioned among populations, and N_m = Wright's gene flow estimate [$N_m = (1\text{-}G_{ST}) / 4G_{ST}$]. Numbers of sampled populations / individuals are given in brackets.

Species	H_T	H_S	D_{ST}	G_{ST}	N_m
N. canadensis [22 / 325]	0.1361	0.0790	0.0572	0.4199	0.3454
N. floridanus [8 / 110]	0.1301	0.0449	0.0853	0.6553	0.1315
N. texanus [20 / 214]	0.2422	0.0756	0.1666	0.6879	0.1134

Table 5. Observed and expected frequencies of heterozygotes across 14 polymorphic loci in three species of *Nuttallanthus*. n = number of sampled individuals.

	N. canadensis		*N. floridanus*		*N. texanus*	
	n = 325		n = 110		n = 214	
Locus	Obs.	Exp.	Obs.	Exp.	Obs.	Exp.
AAT-1	0.0	0.0	0.0	0.0	0.0	0.0975
AAT-2	0.0154	0.3646	0.0	0.0180	0.0047	0.3102
ADH-1	0.0	0.0539	0.0	0.4628	0.0	0.0980
GPD	0.0	0.0713	0.0	0.0	0.0	0.4807
IDH-1	0.0154	0.2467	0.0091	0.2014	0.0	0.0545
MDH-1	0.0	0.0596	0.0	0.1040	0.0	0.4686
MDH-4	0.0	0.0	0.0	0.0701	0.0	0.0
6PGD-2	0.0	0.0	0.0	0.0180	0.0	0.0
PGI-1	0.0	0.0423	0.0	0.0	0.0	0.0
PGI-2	0.0277	0.2788	0.0	0.5902	0.0280	0.3272
PGM-1	0.0	0.0303	0.0	0.1653	0.0	0.4470
SOD	0.0062	0.3921	0.0	0.0	0.0	0.0
TPI-1	0.0	0.0	0.0	0.0	0.0187	0.3107
TPI-2	0.0215	0.4200	0.0	0.0874	0.0	0.6666
Mean	0.0062	0.1400	0.0007	0.1227	0.0037	0.2329
Ratio O/E	0.0443		0.0057		0.0159	

Table 6. Wright's (1978) F statistics averaged across all populations of three species of *Nuttallanthus*. n = number of sampled individuals. Dashes indicate loci that were monomorphic in all sampled populations of a species.

	N. canadensis n = 325			*N. floridanus* n = 110			*N. texanus* n = 214		
Locus	F_{IS}	F_{IT}	F_{ST}	F_{IS}	F_{IT}	F_{ST}	F_{IS}	F_{IT}	F_{ST}
AAT-1	----	----	----	----	----	----	1.000	1.000	0.317
AAT-2	0.908	0.961	0.578	1.000	1.000	0.111	0.954	0.984	0.653
ADH-1	1.000	1.000	0.177	1.000	1.000	0.771	1.000	1.000	0.299
GPD	1.000	1.000	0.201	----	----	----	1.000	1.000	0.912
IDH-1	0.863	0.913	0.370	0.760	0.955	0.814	1.000	1.000	0.350
MDH-1	1.000	1.000	0.191	1.000	1.000	0.183	1.000	1.000	0.459
MDH-4	----	----	----	1.000	1.000	0.143	----	----	----
6PGD-2	----	----	----	1.000	1.000	0.089	----	----	----
PGI-1	1.000	1.000	0.144	----	----	----	----	----	----
PGI-2	0.896	0.913	0.166	1.000	1.000	0.777	0.870	0.921	0.393
PGM-1	1.000	1.000	0.193	1.000	1.000	0.368	1.000	1.000	0.870
SOD	0.965	0.991	0.748	----	----	----	----	----	----
TPI-1	----	----	----	----	----	----	----	----	----
TPI-2	0.929	0.952	0.321	1.000	1.000	0.214	1.000	1.000	0.826
Mean	0.928	0.959	0.430	0.986	0.995	0.640	0.955	0.986	0.694

Table 7. Intra- and interspecific seed production in three species of *Nuttallanthus* following controlled pollinations.

The mean number of seeds produced per capsule is given, followed by the standard deviation in parentheses; n = number of capsules examined per treatment. Seed production varied significantly among all treatments within each species except self hand pollination and intraspecific hand pollination. Individuals of *N. floridanus* produced significantly fewer seeds per capsule for all treatments compared to individuals of *N. canadensis* and *N. texanus*.

				Pollen source for hand pollinations			
					Outcrossing treatments		
Pollen recipient	Cleistogamous untreated	Chasmogamous untreated	Emasculated	Self pollen	*N. canadensis*	*N. floridanus*	*N. texanus*
N. canadensis	148.7 (39.0) n = 100	183.1 (36.6) n = 100	0.0 n = 50	130.7 (33.6) n = 100	120.9 (34.8) n = 75	0.0 n = 30	0.0 n = 35
N. floridanus	107.5 (34.0) n = 100	128.3 (28.6) n = 100	0.0 n = 50	96.1 (31.6) n = 100	0.0 n = 50	97.2 (33.2) n = 75	0.0 n = 50
N. texanus	157 (41.0) n = 100	191.1 (46.5) n = 100	0.0 n = 50	138.3 (47.2) n = 100	0.0 n = 45	0.0 n = 30	125.7 (45.9) n = 80

Figure 1. Numbered collection localities for 60 populations representing three species of *Nuttallanthus* examined for isozyme variation. Collection data are listed in Table 1. Filled symbols (▲●◆) refer to populations included in population-level isozyme analyses; hollow symbols (△○◇) refer to populations sampled but not included in population-level isozyme analyses due to small sample size. Population 58 was divided into two subpopulations (58a and 58b) for analysis.

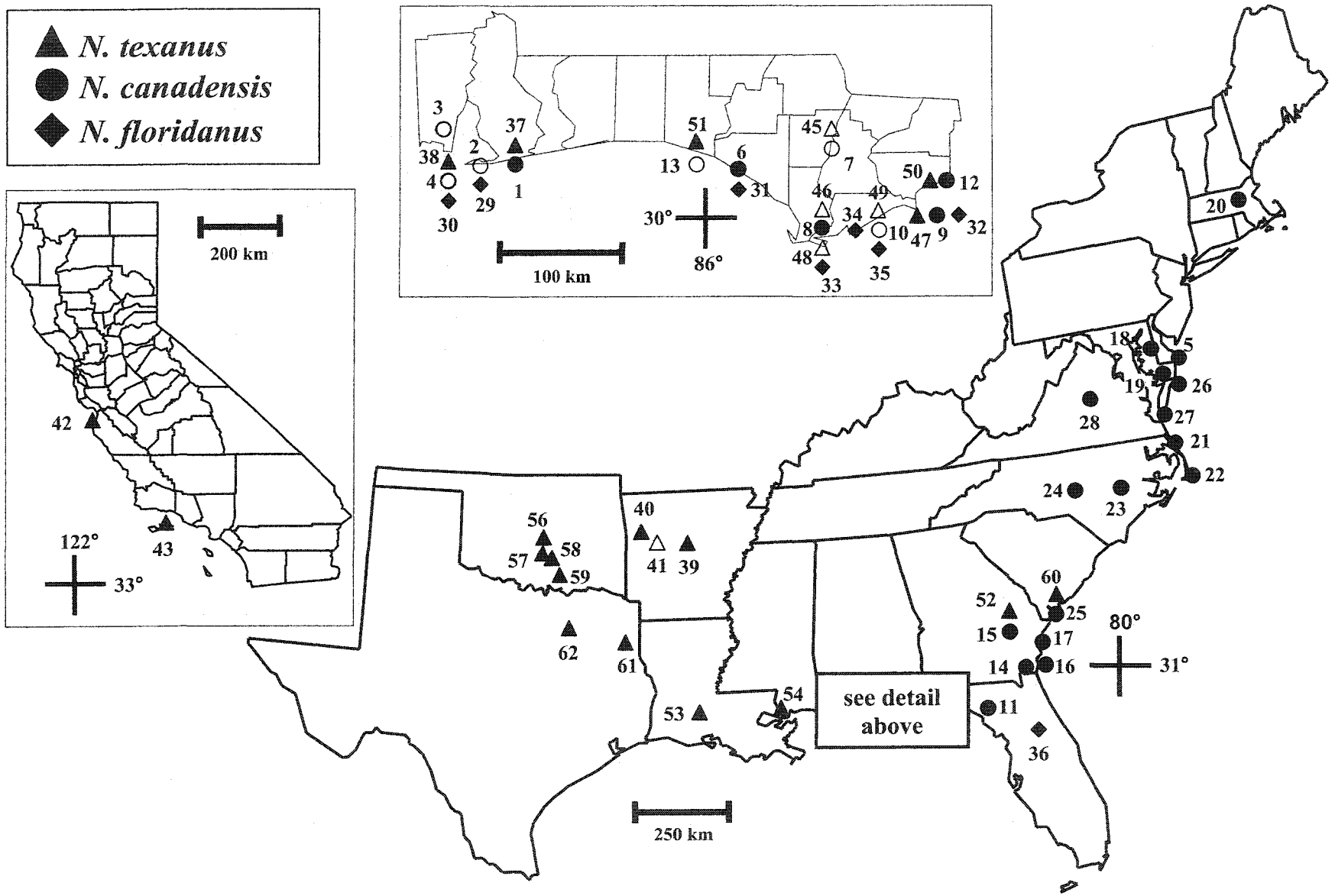

N. texanus
N. canadensis
N. floridanus
200 km
122°
33°
42
43
100 km
30°
86°
3
37
51
45
38
2
13
6
7
50
12
4
29
1
31
46
49
30
34
10
47
9
32
8
48
33
35
see detail above
250 km
80°
31°
20
18
5
19
26
28
27
21
22
24
23
40
56
58
57
41
39
59
60
52
25
15
17
14
16
62
61
53
54
11
36

Figure 2. UPGMA phenogram derived from a matrix of Nei's (1972) genetic identity coefficients among 50 populations of three species of *Nuttallanthus*. Population numbers are listed in Table 1; population localities are depicted in Figure 1. The abbreviations *tex, can* and *flor* refer to populations of *N. texanus, N. canadensis* and *N. floridanus,* respectively, and are followed by the standard two-letter abbreviation for the state of collection. The cophenetic correlation is 0.920.

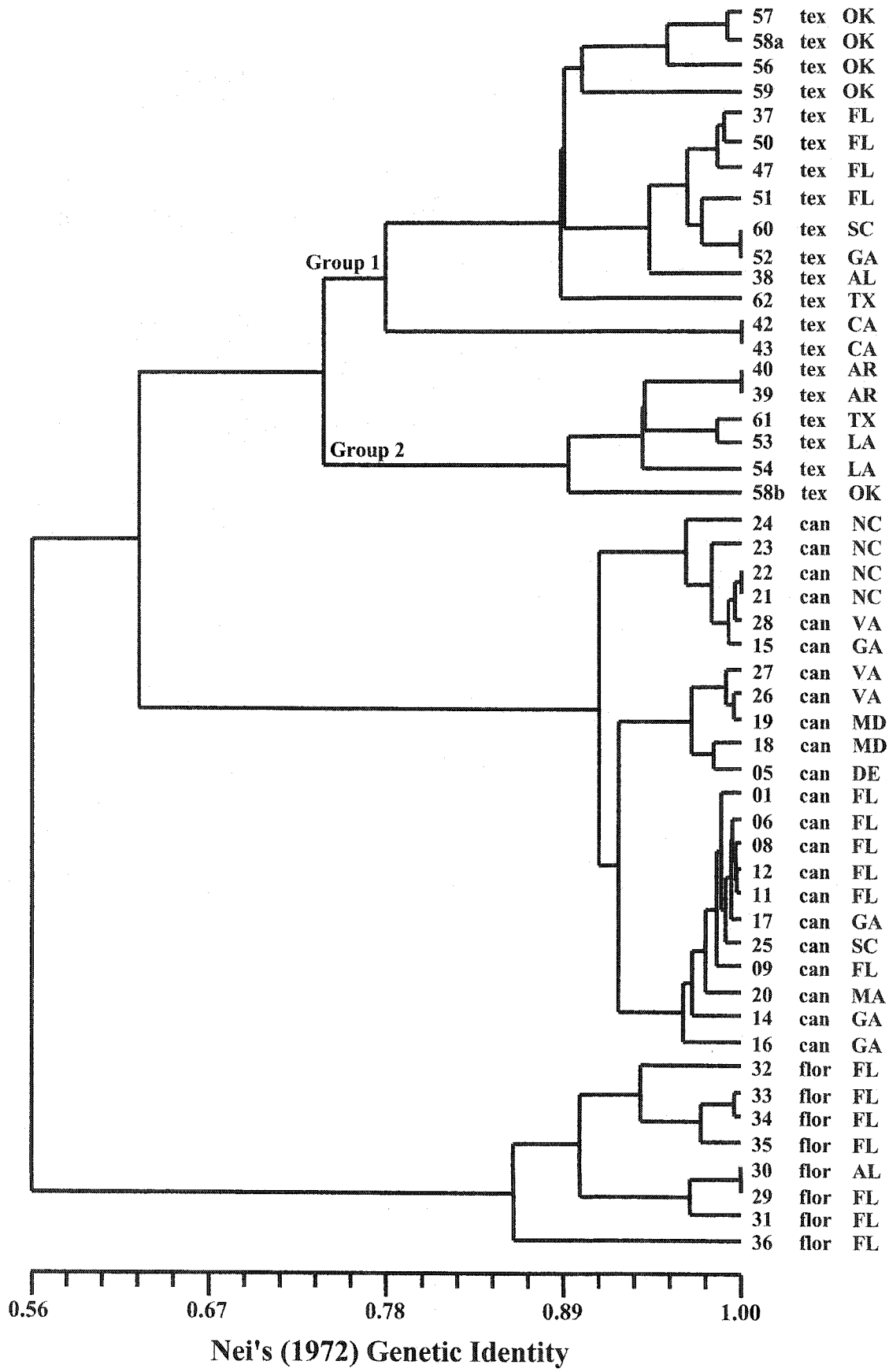
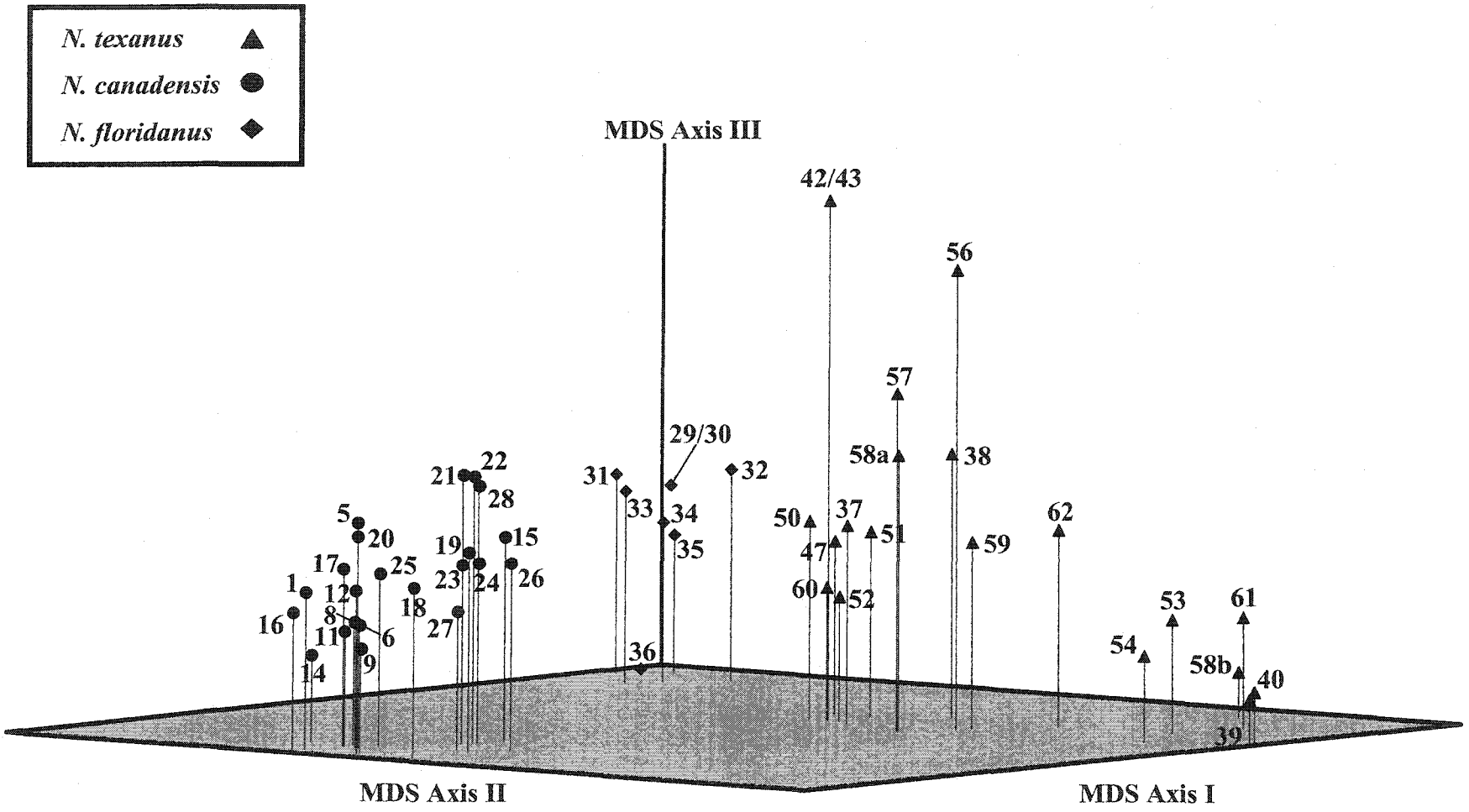
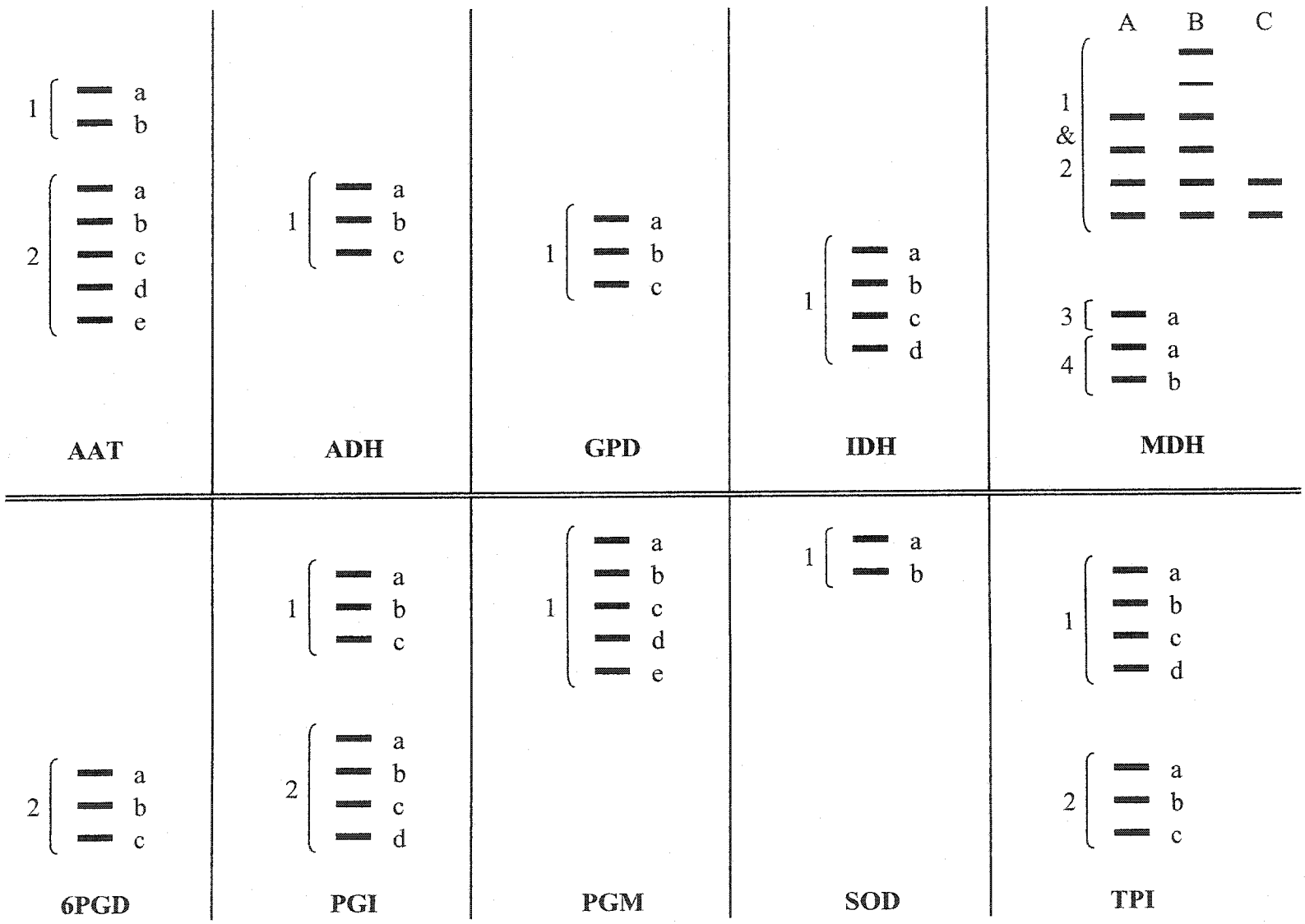

57 tex OK
58a tex OK
56 tex OK
59 tex OK
37 tex FL
50 tex FL
47 tex FL
51 tex FL
60 tex SC
52 tex GA
38 tex AL
62 tex TX
42 tex CA
43 tex CA
40 tex AR
39 tex AR
61 tex TX
53 tex LA
54 tex LA
58b tex OK
24 can NC
23 can NC
22 can NC
21 can NC
28 can VA
15 can GA
27 can VA
26 can VA
19 can MD
18 can MD
05 can DE
01 can FL
06 can FL
08 can FL
12 can FL
11 can FL
17 can GA
25 can SC
09 can FL
20 can MA
14 can GA
16 can GA
32 flor FL
33 flor FL
34 flor FL
35 flor FL
30 flor AL
29 flor FL
31 flor FL
36 flor FL
Group 1
Group 2
0.56
0.67
0.78
0.89
1.00
Nei's (1972) Genetic Identity

Figure 3. Three-dimensional plot of non-metric multidimensional scaling (MDS) analysis of Nei's (1972) genetic identity coefficients among 50 populations of three *Nuttallanthus* species. Population numbers are listed in Table 1; population localities are depicted in Figure 1. Final stress = 0.122.


N. texanus
N. canadensis
N. floridanus
MDS Axis III
MDS Axis II
MDS Axis I
42/43
56
57
29/30
58a
38
32
31
33
34
35
36
50
37
51
47
60
52
59
62
53
61
54
58b
40
39
1
16
14
17
12
8
11
6
9
5
20
25
18
21
22
28
19
23
24
27
15
26

Appendix 1. Diagrams of composite banding patterns of all alleles of loci scored for use in an analysis of genetic variation in three *Nuttallanthus* species. Banding patterns for *MDH-1* and *MDH-2* overlapped too extensively for separate loci and alleles to be reliably distinguished; *MDH-1* and *MDH-2* were treated collectively as a single polymorphic locus with patterns A, B and C corresponding to alleles. *MDH-3* was monomorphic in all individuals examined.


1
a
b
2
a
b
c
d
e
AAT
1
a
b
c
ADH
1
a
b
c
GPD
1
a
b
c
d
IDH
A
B
C
1 & 2
3
a
4
a
b
MDH
1
a
b
2
a
b
c
6PGD
1
a
b
c
2
a
b
c
d
PGI
1
a
b
c
d
e
PGM
1
a
b
SOD
1
a
b
c
d
2
a
b
c
TPI

Appendix 2. Genotype data for loci from 60 populations of *Nuttallanthus canadensis, N. floridanus* and *N. texanus*. Population numbers refer to Table 1; those followed by an asterisk (*) are included in population-level isozyme analyses.

Locus/ genotype	1*	2	3	4	5*	6*	7	8*	9*
AAT-1									
aa	16	5	10	2	30	25	8	19	13
bb	-------	-------	-------	-------	-------	-------	-------	-------	-------
AAT-2									
aa	-------	1	3	-------	14	-------	-------	-------	-------
ab	-------	-------	-------	-------	2	-------	-------	-------	-------
ac	-------	-------	-------	-------	-------	-------	-------	-------	-------
bb	16	4	7	2	14	24	8	19	13
cc	-------	-------	-------	-------	-------	-------	-------	-------	-------
dd	-------	-------	-------	-------	-------	-------	-------	-------	-------
ee	-------	-------	-------	-------	-------	1	-------	-------	-------
ADH-1									
aa	4	-------	3	1	-------	-------	-------	3	2
bb	12	5	7	1	30	25	8	16	11
cc	-------	-------	-------	-------	-------	-------	-------	-------	-------
GPD									
aa	2	-------	2	-------	-------	3	2	2	4
bb	14	5	8	2	30	22	6	17	8
cc	-------	-------	-------	-------	-------	-------	-------	-------	1
IDH-1									
aa	-------	-------	-------	-------	18	-------	-------	-------	-------
ab	-------	-------	-------	-------	-------	-------	-------	-------	-------
bb	10	5	8	2	12	22	8	19	13
bd	1	-------	-------	-------	-------	-------	-------	-------	-------
cc	3	-------	2	-------	-------	3	-------	-------	-------
dd	2	-------	-------	-------	-------	-------	-------	-------	-------
MDH-1									
aa	-------	-------	-------	-------	-------	-------	-------	-------	-------
bb	13	5	10	2	30	22	8	18	13
cc	3	-------	-------	-------	-------	3	-------	1	-------
MDH-4									
aa	-------	-------	-------	-------	-------	-------	-------	-------	-------
bb	16	5	10	2	30	25	8	19	13

Appendix 2 cont.

Locus/ genotype	1*	2	3	4	5*	6*	7	8*	9*
6PGD-2									
aa	-------	-------	-------	-------	-------	-------	-------	-------	-------
bb	-------	-------	-------	-------	-------	-------	-------	-------	-------
cc	16	5	10	2	30	25	8	19	13
PGI-1									
aa	-------	-------	-------	-------	-------	-------	-------	-------	-------
bb	16	5	10	2	30	25	8	19	10
cc	-------	-------	-------	-------	-------	-------	-------	-------	3
PGI-2									
aa	4	-------	2	-------	8	2	-------	2	3
ab	-------	-------	-------	-------	1	-------	-------	1	-------
bb	12	5	8	2	21	23	8	16	10
bc	-------	-------	-------	-------	-------	-------	-------	-------	-------
cc	-------	-------	-------	-------	-------	-------	-------	-------	-------
dd	-------	-------	-------	-------	-------	-------	-------	-------	-------
PGM-1									
aa	-------	-------	-------	-------	-------	-------	-------	-------	-------
bb	-------	-------	-------	-------	-------	-------	-------	-------	-------
cc	16	5	10	2	30	20	8	19	13
dd	-------	-------	-------	-------	-------	-------	-------	-------	-------
ee	-------	-------	-------	-------	-------	5	-------	-------	-------
SOD									
aa	16	1	4	2	29	25	8	18	13
ab	-------	-------	-------	-------	1	-------	-------	-------	-------
bb	-------	4	6	-------	-------	-------	-------	1	-------
TPI-1									
aa	-------	-------	-------	-------	-------	-------	-------	-------	-------
bb	-------	-------	-------	-------	-------	-------	-------	-------	-------
bc	-------	-------	-------	-------	-------	-------	-------	-------	-------
cc	-------	-------	-------	-------	-------	-------	-------	-------	-------
dd	16	5	10	2	30	25	8	19	13
TPI-2									
aa	-------	-------	-------	-------	-------	-------	-------	-------	-------
bb	9	5	9	1	30	13	5	7	8
bc	-------	-------	-------	-------	-------	2	-------	2	-------
cc	7	-------	1	1	-------	10	3	10	5

Appendix 2 cont.

Locus/ genotype	10	11*	12*	13	14*	15*	16*	17*	18*
AAT-1									
aa	3	10	10	5	20	10	10	10	10
bb	-------	-------	-------	-------	-------	-------	-------	-------	-------
AAT-2									
aa	-------	-------	-------	-------	-------	3	-------	-------	8
ab	-------	-------	-------	-------	-------	-------	-------	-------	1
ac	-------	-------	-------	-------	-------	-------	-------	-------	-------
bb	3	10	10	5	20	7	10	10	1
cc	-------	-------	-------	-------	-------	-------	-------	-------	-------
dd	-------	-------	-------	-------	-------	-------	-------	-------	-------
ee	-------	-------	-------	-------	-------	-------	-------	-------	-------
ADH-1									
aa	-------	-------	-------	-------	-------	-------	-------	-------	-------
bb	3	10	10	5	20	10	10	10	10
cc	-------	-------	-------	-------	-------	-------	-------	-------	-------
GPD									
aa	-------	-------	-------	-------	-------	-------	-------	-------	-------
bb	3	10	10	5	20	10	10	10	10
cc	-------	-------	-------	-------	-------	-------	-------	-------	-------
IDH-1									
aa	-------	-------	-------	-------	-------	-------	-------	-------	4
ab	-------	-------	-------	-------	-------	-------	-------	-------	-------
bb	3	9	10	5	18	10	2	10	6
bd	-------	-------	-------	-------	-------	-------	3	-------	-------
cc	-------	-------	-------	-------	-------	-------	-------	-------	-------
dd	-------	1	-------	-------	2	-------	5	-------	-------
MDH-1									
aa	-------	-------	-------	-------	-------	-------	-------	-------	-------
bb	3	10	10	4	20	10	10	10	10
cc	-------	-------	-------	1	-------	-------	-------	-------	-------
MDH-4									
aa	-------	-------	-------	-------	-------	-------	-------	-------	-------
bb	3	10	10	5	20	10	10	10	10

Appendix 2 cont.

Locus/ genotype	10	11*	12*	13	14*	15*	16*	17*	18*
6PGD-2									
aa	-------	-------	-------	-------	-------	-------	-------	-------	-------
bb	-------	-------	-------	-------	-------	-------	-------	-------	-------
cc	3	10	10	5	20	10	10	10	10
PGI-1									
aa	-------	-------	-------	-------	-------	-------	-------	-------	-------
bb	3	10	10	5	19	10	10	10	9
cc	-------	-------	-------	-------	1	-------	-------	-------	1
PGI-2									
aa	-------	-------	-------	-------	4	-------	-------	3	-------
ab	-------	-------	-------	-------	3	-------	-------	-------	-------
bb	3	10	10	5	13	9	10	7	10
bc	-------	-------	-------	-------	-------	-------	-------	-------	-------
cc	-------	-------	-------	-------	-------	1	-------	-------	-------
dd	-------	-------	-------	-------	-------	-------	-------	-------	-------
PGM-1									
aa	-------	-------	-------	-------	-------	-------	-------	-------	-------
bb	-------	-------	-------	-------	-------	-------	-------	-------	-------
cc	3	10	10	5	20	10	10	10	10
dd	-------	-------	-------	-------	-------	-------	-------	-------	-------
ee	-------	-------	-------	-------	-------	-------	-------	-------	-------
SOD									
aa	3	10	10	5	20	1	10	10	10
ab	-------	-------	-------	-------	-------	-------	-------	-------	-------
bb	-------	-------	-------	-------	-------	9	-------	-------	-------
TPI-1									
aa	-------	-------	-------	-------	-------	-------	-------	-------	-------
bb	-------	-------	-------	-------	-------	-------	-------	-------	-------
bc	-------	-------	-------	-------	-------	-------	-------	-------	-------
cc	-------	-------	-------	-------	-------	-------	-------	-------	-------
dd	3	10	10	5	20	10	10	10	10
TPI-2									
aa	-------	-------	-------	-------	-------	-------	-------	-------	-------
bb	-------	3	6	5	1	9	6	6	9
bc	-------	1	-------	-------	-------	-------	-------	-------	-------
cc	3	6	4	-------	19	1	4	4	1

Appendix 2 cont.

Locus/ genotype	19*	20*	21*	22*	23*	24*	25*	26*	27*
AAT-1									
aa	17	15	10	10	10	20	10	20	20
bb	-------	-------	-------	-------	-------	-------	-------	-------	-------
AAT-2									
aa	11	-------	-------	-------	3	3	-------	18	14
ab	1	-------	-------	-------	-------	1	-------	-------	-------
ac	-------	-------	-------	-------	-------	-------	-------	-------	-------
bb	5	15	10	10	7	16	10	2	6
cc	-------	-------	-------	-------	-------	-------	-------	-------	-------
dd	-------	-------	-------	-------	-------	-------	-------	-------	-------
ee	-------	-------	-------	-------	-------	-------	-------	-------	-------
ADH-1									
aa	-------	-------	-------	-------	-------	-------	-------	-------	-------
bb	17	15	10	10	10	20	10	20	20
cc	-------	-------	-------	-------	-------	-------	-------	-------	-------
GPD									
aa	-------	-------	-------	-------	-------	-------	-------	-------	-------
bb	17	15	10	10	10	20	10	20	20
cc	-------	-------	-------	-------	-------	-------	-------	-------	-------
IDH-1									
aa	2	-------	-------	-------	2	-------	-------	-------	-------
ab	1	-------	-------	-------	-------	-------	-------	-------	-------
bb	14	15	10	10	8	20	10	20	20
bd	-------	-------	-------	-------	-------	-------	-------	-------	-------
cc	-------	-------	-------	-------	-------	-------	-------	-------	-------
dd	-------	-------	-------	-------	-------	-------	-------	-------	-------
MDH-1									
aa	-------	-------	-------	-------	-------	-------	-------	-------	-------
bb	17	15	10	10	10	20	7	20	20
cc	-------	-------	-------	-------	-------	-------	3	-------	-------
MDH-4									
aa	-------	-------	-------	-------	-------	-------	-------	-------	-------
bb	17	15	10	10	10	20	10	20	20

Appendix 2 cont.

Locus/ genotype	19*	20*	21*	22*	23*	24*	25*	26*	27*
6PGD-2									
aa	-------	-------	-------	-------	-------	-------	-------	-------	-------
bb	-------	-------	-------	-------	-------	-------	-------	-------	-------
cc	17	15	10	10	10	20	10	20	20
PGI-1									
aa	2	-------	-------	-------	-------	-------	-------	-------	-------
bb	15	15	10	10	10	20	10	20	20
cc	-------	-------	-------	-------	-------	-------	-------	-------	-------
PGI-2									
aa	1	-------	1	2	2	11	-------	1	1
ab	-------	-------	-------	1	-------	-------	-------	1	2
bb	16	15	7	7	8	9	10	17	17
bc	-------	-------	-------	-------	-------	-------	-------	-------	-------
cc	-------	-------	2	-------	-------	-------	-------	1	-------
dd	-------	-------	-------	-------	-------	-------	-------	-------	-------
PGM-1									
aa	-------	-------	-------	-------	-------	-------	-------	-------	-------
bb	-------	-------	-------	-------	-------	-------	-------	-------	-------
cc	17	15	10	10	10	20	10	20	20
dd	-------	-------	-------	-------	-------	-------	-------	-------	-------
ee	-------	-------	-------	-------	-------	-------	-------	-------	-------
SOD									
aa	11	15	-------	-------	4	-------	8	11	16
ab	-------	-------	-------	-------	-------	-------	-------	-------	1
bb	6	-------	10	10	6	20	2	9	3
TPI-1									
aa	-------	-------	-------	-------	-------	-------	-------	-------	-------
bb	-------	-------	-------	-------	-------	-------	-------	-------	-------
bc	-------	-------	-------	-------	-------	-------	-------	-------	-------
cc	-------	-------	-------	-------	-------	-------	-------	-------	-------
dd	17	15	10	10	10	20	10	20	20
TPI-2									
aa	-------	-------	-------	-------	-------	-------	-------	-------	-------
bb	17	15	10	10	7	9	6	19	14
bc	-------	-------	-------	-------	1	-------	-------	-------	1
cc	-------	-------	-------	-------	2	11	4	1	5

Appendix 2 cont.

Locus/ genotype	28*	29*	30*	31*	32*	33*	34*	35*	36*
AAT-1									
aa	10	11	11	25	10	8	20	10	15
bb	-------	-------	-------	-------	-------	-------	-------	-------	-------
AAT-2									
aa	-------	-------	-------	-------	-------	-------	-------	-------	-------
ab	-------	-------	-------	-------	-------	-------	-------	-------	-------
ac	-------	-------	-------	-------	-------	-------	-------	-------	-------
bb	10	-------	-------	-------	-------	1	-------	-------	-------
cc	-------	11	11	25	10	7	20	10	15
dd	-------	-------	-------	-------	-------	-------	-------	-------	-------
ee	-------	-------	-------	-------	-------	-------	-------	-------	-------
ADH-1									
aa	-------	-------	-------	-------	-------	-------	-------	-------	-------
bb	10	-------	-------	-------	9	7	20	4	-------
cc	-------	11	11	25	1	1	-------	6	15
GPD									
aa	-------	-------	-------	-------	-------	-------	-------	-------	-------
bb	10	11	11	25	10	8	20	10	15
cc	-------	-------	-------	-------	-------	-------	-------	-------	-------
IDH-1									
aa	-------	-------	-------	-------	-------	-------	-------	-------	-------
ab	-------	-------	-------	-------	-------	-------	-------	-------	-------
bb	10	11	11	25	10	8	20	10	2
bd	-------	-------	-------	-------	-------	-------	-------	-------	1
cc	-------	-------	-------	-------	-------	-------	-------	-------	-------
dd	-------	-------	-------	-------	-------	-------	-------	-------	12
MDH-1									
aa	-------	-------	-------	-------	3	-------	-------	-------	2
bb	10	11	11	25	7	8	19	10	13
cc	-------	-------	-------	-------	-------	-------	1	-------	-------
MDH-4									
aa	-------	-------	-------	4	-------	-------	-------	-------	-------
bb	10	11	11	21	10	8	20	10	15

Appendix 2 cont.

Locus/ genotype	28*	29*	30*	31*	32*	33*	34*	35*	36*
6PGD-2									
aa	-------	-------	-------	-------	1	-------	-------	-------	-------
bb	-------	11	11	25	9	8	20	10	15
cc	10	-------	-------	-------	-------	-------	-------	-------	-------
PGI-1									
aa	-------	-------	-------	-------	-------	-------	-------	-------	-------
bb	10	11	11	25	10	8	20	10	15
cc	-------	-------	-------	-------	-------	-------	-------	-------	-------
PGI-2									
aa	-------	-------	-------	-------	-------	-------	-------	-------	-------
ab	-------	-------	-------	-------	-------	-------	-------	-------	-------
bb	10	-------	-------	-------	-------	-------	-------	-------	13
bc	-------	-------	-------	-------	-------	-------	-------	-------	-------
cc	-------	-------	-------	13	2	8	20	10	2
dd	-------	11	11	12	8	-------	-------	-------	-------
PGM-1									
aa	-------	-------	-------	-------	-------	-------	-------	-------	-------
bb	-------	-------	-------	-------	-------	-------	-------	-------	-------
cc	10	-------	-------	10	-------	-------	-------	-------	-------
dd	-------	11	11	15	10	8	20	10	15
ee	-------	-------	-------	-------	-------	-------	-------	-------	-------
SOD									
aa	-------	-------	-------	-------	-------	-------	-------	-------	-------
ab	-------	-------	-------	-------	-------	-------	-------	-------	-------
bb	10	11	11	25	10	8	20	10	15
TPI-1									
aa	-------	-------	-------	-------	-------	-------	-------	-------	-------
bb	-------	-------	-------	-------	-------	-------	-------	-------	-------
bc	-------	-------	-------	-------	-------	-------	-------	-------	-------
cc	-------	11	11	25	10	8	20	10	15
dd	10	-------	-------	-------	-------	-------	-------	-------	-------
TPI-2									
aa	-------	-------	-------	-------	-------	1	-------	-------	-------
bb	10	-------	-------	-------	-------	2	2	-------	-------
bc	-------	-------	-------	-------	-------	-------	-------	-------	-------
cc	-------	11	11	25	10	5	18	10	15

Appendix 2 cont.

Locus/ genotype	37*	38*	39*	40*	41	42*	43*	45	46
AAT-1									
aa	14	10	10	10	5	5	5	4	1
bb	-------	-------	-------	-------	-------	-------	-------	-------	-------
AAT-2									
aa	2	-------	10	10	3	-------	-------	-------	-------
ab	-------	-------	-------	-------	-------	-------	-------	-------	-------
ac	-------	-------	-------	-------	-------	-------	-------	-------	-------
bb	-------	-------	-------	-------	-------	-------	-------	-------	-------
cc	12	9	-------	-------	2	5	5	4	1
dd	-------	1	-------	-------	-------	-------	-------	-------	-------
ee	-------	-------	-------	-------	-------	-------	-------	-------	-------
ADH-1									
aa	-------	-------	-------	-------	-------	-------	-------	1	-------
bb	14	10	10	10	5	5	5	3	1
cc	-------	-------	-------	-------	-------	-------	-------	-------	-------
GPD									
aa	-------	-------	10	10	1	-------	-------	-------	-------
bb	14	10	-------	-------	4	5	5	4	1
cc	-------	-------	-------	-------	-------	-------	-------	-------	-------
IDH-1									
aa	-------	-------	-------	-------	-------	-------	-------	-------	-------
ab	-------	-------	-------	-------	-------	-------	-------	-------	-------
bb	13	10	10	10	5	5	5	4	1
bd	-------	-------	-------	-------	-------	-------	-------	-------	-------
cc	1	-------	-------	-------	-------	-------	-------	-------	-------
dd	-------	-------	-------	-------	-------	-------	-------	-------	-------
MDH-1									
aa	6	10	-------	-------	-------	-------	-------	2	1
bb	8	-------	10	10	5	5	5	2	-------
cc	-------	-------	-------	-------	-------	-------	-------	-------	-------
MDH-4									
aa	-------	-------	-------	-------	-------	-------	-------	-------	-------
bb	14	10	10	10	5	5	5	4	1

Appendix 2 cont.

Locus/ genotype	37*	38*	39*	40*	41	42*	43*	45	46
6PGD-2									
aa	14	10	10	10	5	5	5	4	1
bb	-------	-------	-------	-------	-------	-------	-------	-------	-------
cc	-------	-------	-------	-------	-------	-------	-------	-------	-------
PGI-1									
aa	-------	-------	-------	-------	-------	-------	-------	-------	-------
bb	14	10	10	10	5	5	5	4	1
cc	-------	-------	-------	-------	-------	-------	-------	-------	-------
PGI-2									
aa	3	-------	-------	1	-------	-------	-------	1	-------
ab	-------	-------	-------	-------	1	-------	-------	-------	-------
bb	11	10	10	9	4	5	5	2	1
bc	-------	-------	-------	-------	-------	-------	-------	-------	-------
cc	-------	-------	-------	-------	-------	-------	-------	1	-------
dd	-------	-------	-------	-------	-------	-------	-------	-------	-------
PGM-1									
aa	-------	-------	10	10	5	-------	-------	-------	-------
bb	-------	-------	-------	-------	-------	5	5	-------	-------
cc	14	10	-------	-------	-------	-------	-------	4	1
dd	-------	-------	-------	-------	-------	-------	-------	-------	-------
ee	-------	-------	-------	-------	-------	-------	-------	-------	-------
SOD									
aa	-------	-------	-------	-------	-------	-------	-------	-------	-------
ab	-------	-------	-------	-------	-------	-------	-------	-------	-------
bb	14	10	10	10	5	5	5	4	1
TPI-1									
aa	-------	-------	-------	-------	-------	5	5	-------	-------
bb	11	10	10	10	5	-------	-------	2	1
bc	-------	-------	-------	-------	-------	-------	-------	-------	-------
cc	3	-------	-------	-------	-------	-------	-------	2	-------
dd	-------	-------	-------	-------	-------	-------	-------	-------	-------
TPI-2									
aa	3	-------	10	10	5	-------	-------	-------	-------
bb	3	1	-------	-------	-------	5	5	1	-------
bc	-------	-------	-------	-------	-------	-------	-------	-------	-------
cc	8	9	-------	-------	-------	-------	-------	3	1

Appendix 2 cont.

Locus/ genotype	47*	48	49	50*	51*	52*	53*	54*	56*
AAT-1									
aa	13	5	1	13	7	5	10	12	10
bb	-------	-------	-------	-------	-------	-------	-------	-------	-------
AAT-2									
aa	-------	-------	-------	-------	-------	-------	2	8	-------
ab	-------	-------	-------	-------	-------	-------	-------	-------	-------
ac	-------	-------	-------	-------	-------	-------	1	-------	-------
bb	-------	-------	-------	-------	-------	-------	-------	-------	-------
cc	13	5	1	13	7	5	7	4	10
dd	-------	-------	-------	-------	-------	-------	-------	-------	-------
ee	-------	-------	-------	-------	-------	-------	-------	-------	-------
ADH-1									
aa	-------	-------	-------	3	-------	-------	-------	-------	-------
bb	13	5	1	10	7	5	10	12	10
cc	-------	-------	-------	-------	-------	-------	-------	-------	-------
GPD									
aa	-------	-------	-------	-------	-------	-------	10	12	-------
bb	13	5	1	13	7	5	-------	-------	10
cc	-------	-------	-------	-------	-------	-------	-------	-------	-------
IDH-1									
aa	-------	-------	-------	-------	-------	-------	-------	-------	-------
ab	-------	-------	-------	-------	-------	-------	-------	-------	-------
bb	13	5	1	13	7	5	10	7	10
bd	-------	-------	-------	-------	-------	-------	-------	-------	-------
cc	-------	-------	-------	-------	-------	-------	-------	5	-------
dd	-------	-------	-------	-------	-------	-------	-------	-------	-------
MDH-1									
aa	8	1	-------	4	1	-------	-------	-------	9
bb	5	4	1	9	6	5	8	11	1
cc	-------	-------	-------	-------	-------	-------	2	1	-------
MDH-4									
aa	-------	-------	-------	-------	-------	-------	-------	-------	-------
bb	13	5	1	13	7	5	10	12	10

Appendix 2 cont.

Locus/ genotype	47*	48	49	50*	51*	52*	53*	54*	56*
6PGD-2									
aa	13	5	1	13	7	5	10	12	10
bb	-------	-------	-------	-------	-------	-------	-------	-------	-------
cc	-------	-------	-------	-------	-------	-------	-------	-------	-------
PGI-1									
aa	-------	-------	-------	-------	-------	-------	-------	-------	-------
bb	13	5	1	13	7	5	10	12	10
cc	-------	-------	-------	-------	-------	-------	-------	-------	-------
PGI-2									
aa	-------	1	-------	-------	4	-------	-------	-------	8
ab	-------	-------	-------	-------	1	-------	-------	-------	-------
bb	12	4	1	13	2	5	10	9	2
bc	-------	-------	-------	-------	-------	-------	-------	2	-------
cc	1	-------	-------	-------	-------	-------	-------	1	-------
dd	-------	-------	-------	-------	-------	-------	-------	-------	-------
PGM-1									
aa	-------	-------	-------	-------	-------	-------	6	3	-------
bb	-------	-------	-------	-------	-------	-------	-------	-------	-------
cc	13	5	1	13	7	5	4	9	10
dd	-------	-------	-------	-------	-------	-------	-------	-------	-------
ee	-------	-------	-------	-------	-------	-------	-------	-------	-------
SOD									
aa	-------	-------	-------	-------	-------	-------	-------	-------	-------
ab	-------	-------	-------	-------	-------	-------	-------	-------	-------
bb	13	5	1	13	7	5	10	12	10
TPI-1									
aa	-------	-------	-------	-------	-------	-------	-------	-------	-------
bb	7	5	1	7	7	5	10	12	10
bc	-------	-------	-------	2	-------	-------	-------	-------	-------
cc	6	-------	-------	4	-------	-------	-------	-------	-------
dd	-------	-------	-------	-------	-------	-------	-------	-------	-------
TPI-2									
aa	-------	1	-------	-------	-------	-------	10	12	-------
bb	-------	3	-------	3	-------	-------	-------	-------	10
bc	-------	-------	-------	-------	-------	-------	-------	-------	-------
cc	13	1	1	10	7	5	-------	-------	-------

Appendix 2 cont.

Locus/ genotype	57*	58a*	58b*	59*	60*	61*	62*
AAT-1							
aa	12	10	12	10	10	10	15
bb	8	3	-------	-------	-------	-------	-------
AAT-2							
aa	-------	2	-------	4	-------	-------	-------
ab	-------	-------	-------	-------	-------	-------	-------
ac	-------	-------	-------	-------	-------	-------	-------
bb	-------	-------	-------	-------	-------	-------	-------
cc	20	11	12	6	10	9	15
dd	-------	-------	-------	-------	-------	1	-------
ee	-------	-------	-------	-------	-------	-------	-------
ADH-1							
aa	-------	3	-------	4	-------	-------	-------
bb	20	10	12	5	10	10	15
cc	-------	-------	-------	1	-------	-------	-------
GPD							
aa	-------	3	12	4	-------	10	15
bb	20	10	-------	6	10	-------	-------
cc	-------	-------	-------	-------	-------	-------	-------
IDH-1							
aa	-------	-------	-------	-------	-------	-------	-------
ab	-------	-------	-------	-------	-------	-------	-------
bb	20	13	12	10	10	10	15
bd	-------	-------	-------	-------	-------	-------	-------
cc	-------	-------	-------	-------	-------	-------	-------
dd	-------	-------	-------	-------	-------	-------	-------
MDH-1							
aa	10	5	-------	-------	-------	-------	6
bb	10	8	8	6	10	10	9
cc	-------	-------	4	4	-------	-------	-------
MDH-4							
aa	-------	-------	-------	-------	-------	-------	-------
bb	20	13	12	10	10	10	15

Appendix 2 cont.

Locus/ genotype	57*	58a*	58b*	59*	60*	61*	62*
6PGD-2							
aa	20	13	12	10	10	10	15
bb	-------	-------	-------	-------	-------	-------	-------
cc	-------	-------	-------	-------	-------	-------	-------
PGI-1							
aa	-------	-------	-------	-------	-------	-------	-------
bb	20	13	12	10	10	10	15
cc	-------	-------	-------	-------	-------	-------	-------
PGI-2							
aa	4	4	-------	5	1	-------	8
ab	2	-------	-------	-------	-------	-------	-------
bb	14	8	12	5	9	10	7
bc	-------	1	-------	-------	-------	-------	-------
cc	-------	-------	-------	-------	-------	-------	-------
dd	-------	-------	-------	-------	-------	-------	-------
PGM-1							
aa	-------	-------	12	4	-------	10	-------
bb	-------	-------	-------	-------	-------	-------	-------
cc	20	13	-------	6	10	-------	15
dd	-------	-------	-------	-------	-------	-------	-------
ee	-------	-------	-------	-------	-------	-------	-------
SOD							
aa	-------	-------	-------	-------	-------	-------	-------
ab	-------	-------	-------	-------	-------	-------	-------
bb	20	13	12	10	10	10	15
TPI-1							
aa	-------	-------	-------	-------	-------	-------	-------
bb	20	13	1	6	9	10	15
bc	-------	-------	2	-------	-------	-------	-------
cc	-------	-------	9	4	1	-------	-------
dd	-------	-------	-------	-------	-------	-------	-------
TPI-2							
aa	-------	-------	12	4	-------	10	-------
bb	19	12	-------	6	-------	-------	6
bc	-------	-------	-------	-------	-------	-------	-------
cc	1	1	-------	-------	10	-------	9

Appendix 3. Summary allele frequencies for 14 putative polymorphic loci within three species of *Nuttallanthus*. n = number of sampled individuals.

	Nuttallanthus species		
Locus/allele	*N. canadensis* n = 325	*N. floridanus* n = 110	*N. texanus* n = 214
AAT-1			
a	1.000	1.000	0.949
b	----	----	0.051
AAT-2			
a	0.235	----	0.180
b	0.762	0.009	----
c	----	0.991	0.811
d	----	----	0.009
e	0.003	----	----
ADH-1			
a	0.028	----	0.047
b	0.972	0.364	0.949
c	----	0.636	0.005
GPD			
a	0.034	----	0.402
b	0.963	1.000	0.598
c	0.003	----	----
IDH-1			
a	0.082	----	----
b	0.863	0.886	0.972
c	0.019	----	0.028
d	0.037	0.114	----
MDH-1			
a	----	0.045	0.276
b	0.969	0.945	0.673
c	0.031	0.009	0.051

Appendix 3 cont.

MDH-4			
a	----	0.036	----
b	1.000	0.964	1.000
6PGD-2			
a	----	0.009	1.000
b	----	0.991	----
c	1.000	----	----
PGI-1			
a	0.006	----	----
b	0.978	1.000	1.000
c	0.015	----	----
PGI-2			
a	0.152	----	0.185
b	0.835	0.118	0.799
c	0.012	0.500	0.017
d	----	0.382	----
PGM-1			
a	----	----	0.257
b	----	----	0.047
c	0.985	0.091	0.696
d	----	0.909	----
e	0.015	----	----
SOD			
a	0.732	----	----
b	0.268	1.000	1.000
TPI-1			
a	----	----	0.047
b	----	----	0.818
c	----	1.000	0.136
d	1.000	----	----
TPI-2			
a	----	0.009	0.332
b	0.700	0.036	0.327
c	0.300	0.955	0.341

Appendix 4. Wright's fixation indices (F) for 42 populations of three *Nuttallanthus* species, indicating deviation from Hardy-Weinburg genotypic expectations. Significance levels were calculated by pooling genotypes and comparing the frequencies obtained with those expected under random mating. Dashes indicate monomorphic loci. Populations that were monomorphic across all loci are not represented in the table. Population numbers refer to Table 1. *P<0.05; **P<0.01; ***P<0.001

	Populations										
Locus	1	5	6	8	9	11	12	14	15	16	17
AAT-1	---	---	---	---	---	---	---	---	---	---	---
AAT-2	---	0.867***	1.000***	---	---	---	---	---	1.000**	---	---
ADH-1	1.000***	---	---	1.000***	1.000***	---	---	---	---	---	---
GPD	1.000***	---	1.000***	1.000***	1.000***	---	---	---	---	---	---
IDH-1	0.877**	1.000***	1.000***	---	---	1.000**	---	1.000***	---	0.341	---
MDH-1	1.000***	---	1.000***	1.000***	---	---	---	---	---	---	---
MDH-4	---	---	---	---	---	---	---	---	---	---	---
6PGD-2	---	---	---	---	---	---	---	---	---	---	---
PGI-1	---	---	---	---	1.000***	---	---	1.000***	---	---	---
PGI-2	1.000***	0.918***	1.000***	0.770**	1.000***	---	---	0.624**	1.000**	---	1.000**
PGM-1	---	---	1.000***	---	---	---	---	---	---	---	---
SOD	---	-0.017	---	1.000***	---	---	---	---	1.000**	---	---
TPI-1	---	---	---	---	---	---	---	---	---	---	---
TPI-2	1.000***	---	0.838***	0.784**	1.000***	0.780*	1.000**	1.000***	1.000**	1.000**	1.000**

Appendix 4 cont.

Locus	Populations										
	18	19	21	22	23	24	25	26	27	31	32
AAT-1	---	---	---	---	---	---	---	---	---	---	---
AAT-2	0.608	0.866***	---	---	1.000**	0.827***	---	1.000***	1.000***	---	---
ADH-1	---	---	---	---	---	---	---	---	---	---	1.000**
GPD	---	---	---	---	---	---	---	---	---	---	---
IDH-1	1.000**	0.766***	---	---	1.000**	---	---	---	---	---	---
MDH-1	---	---	---	---	---	---	1.000**	---	---	---	1.000**
MDH-4	---	---	---	---	---	---	---	---	---	1.000***	---
6PGD-2	---	---	---	---	---	---	---	---	---	---	1.000**
PGI-1	1.000**	1.000***	---	---	---	---	---	---	---	---	---
PGI-2	---	1.000***	1.000**	0.733*	1.000**	1.000***	---	0.779**	0.444*	1.000***	1.000**
PGM-1	---	---	---	---	---	---	---	---	---	1.000***	---
SOD	---	1.000***	---	---	1.000**	---	1.000**	1.000***	0.827***	---	---
TPI-1	---	---	---	---	---	---	---	---	---	---	---
TPI-2	1.000**	---	---	---	0.733*	1.000***	1.000**	1.000***	0.875***	---	---

Appendix 4 cont.

Locus	Populations										
	33	34	35	36	37	38	40	47	50	51	53
AAT-1	---	---	---	---	---	---	---	---	---	---	---
AAT-2	1.000**	---	---	---	1.000***	1.000**	---	---	---	---	0.733*
ADH-1	1.000**	---	1.000**	---	---	---	---	---	1.000***	---	---
GPD	---	---	---	---	---	---	---	---	---	---	---
IDH-1	---	---	---	0.760**	1.000***	---	---	---	---	---	---
MDH-1	---	1.000***	---	1.000***	1.000***	---	---	1.000***	1.000***	1.000**	1.000**
MDH-4	---	---	---	---	---	---	---	---	---	---	---
6PGD-2	---	---	---	---	---	---	---	---	---	---	---
PGI-1	---	---	---	---	---	---	---	---	---	---	---
PGI-2	---	---	---	1.000***	1.000***	---	1.000**	1.000***	---	0.689	---
PGM-1	---	---	---	---	---	---	---	---	---	---	1.000**
SOD	---	---	---	---	---	---	---	---	---	---	---
TPI-1	---	---	---	---	1.000***	---	---	1.000***	0.675*	---	---
TPI-2	1.000**	1.000***	---	---	1.000***	1.000**	---	---	1.000***	---	---

Appendix 4 cont.

Locus	Populations								
	54	56	57	58a	58b	59	60	61	62
AAT-1	---	---	1.000***	1.000***	---	---	---	---	---
AAT-2	1.000**	---	---	1.000***	---	1.000**	---	1.000**	---
ADH-1	---	---	---	1.000***	---	1.000**	---	---	---
GPD	---	---	---	1.000***	---	1.000**	---	---	---
IDH-1	1.000**	---	---	---	---	---	---	---	---
MDH-1	1.000**	1.000**	1.000***	1.000***	1.000**	1.000**	---	---	1.000***
MDH-4	---	---	---	---	---	---	---	---	---
6PGD-2	---	---	---	---	---	---	---	---	---
PGI-1	---	---	---	---	---	---	---	---	---
PGI-2	0.400	1.000**	0.733**	0.839**	---	1.000**	1.000**	---	1.000***
PGM-1	1.000**	---	---	---	---	1.000**	---	---	---
SOD	---	---	---	---	---	---	---	---	---
TPI-1	---	---	---	---	0.400	1.000**	1.000**	---	---
TPI-2	---	---	1.000***	1.000***	---	1.000**	---	---	1.000***

CHAPTER 2.

MORPHOLOGICAL VARIATION AND SYSTEMATICS OF NORTH AMERICAN SPECIES OF *NUTTALLANTHUS* (LAMIALES)

ABSTRACT

The genus *Nuttallanthus* in North America consists of three currently recognized species of annual or biennial herbs with heteromorphic stems and showy personate and bilabiate flowers. High levels of intraspecific morphological variation and intergradation in the morphological characters used to distinguish among these taxa have led to diverse taxonomic treatments of the members of this group. To assess patterns of morphological variation within and among these taxa and to delineate morphological gaps within the genus, 48 vegetative, floral, fruit and seed characters were measured from field-collected samples acquired from 50 populations of *Nuttallanthus*. Univariate analyses of morphometric data indicated significant variation among taxa in all of the measured traits. Principal component analysis and cluster analysis using individuals and population means as OTUs consistently separated samples into three primary clusters corresponding to the currently circumscribed species; reproductive characters provided much better discrimination than did vegetative characters. Individuals of the three taxa were correctly classified by discriminant function analysis in 79.8% of cases using only vegetative traits and in 100% of cases using only reproductive characters and all characters. Correlation analysis indicated positive and statistically-significant associations between phenotypic distance and linear distance between populations of each species. These results were consistent with analyses of genetic variation in the North American species of *Nuttallanthus*; strong and significant correlations between phenotypic distance and genetic distance were observed among populations and species. Chromosome counts were obtained from each population of the three species represented in this study; all counts were n = 6. The observed morphological discontinuities separating groups in this

genus and the close correspondence of extensive morphological and genetic gaps among these taxa strongly support the recognition of three species in North American *Nuttallanthus*.

INTRODUCTION

Three native North American species of annual or biennial herbaceous plants are currently recognized in the genus *Nuttallanthus*: *N. canadensis* (L.) D. A. Sutton, *N. floridanus* (Chapman) D. A. Sutton and *N. texanus* (Scheele) D. A. Sutton (Sutton 1988, USDA/NRCS 2002).). *Nuttallanthus canadensis* is native to much of North America and is naturalized in temperate regions of South America and in Europe, where it has been cultivated as an ornamental for its showy, fragrant flowers (Tutin et al. 1972). *N. texanus* is native to the southern United States and Mexico, may be native to South America, and is naturalized in other temperate areas (Sutton 1988). *Nuttallanthus floridanus* is more narrowly distributed and occurs in the Atlantic and Gulf coastal plain of a few states in the southeastern U.S.A. Species ranges overlap in southeastern North America where all three taxa occur occasionally in dense, mixed populations. These species are commonly found in sandy soils of open coniferous woodlands, dunes and crop fields; they may occur in a wide variety of subtrates as adventives in heavily disturbed sites. All species of *Nuttallanthus* produce erect fertile stems with simple, linear, alternately-arranged leaves, and sterile prostrate stems with elliptical, verticillate leaves. The strongly zygomorphic, bilabiate flowers are borne on terminal racemes and produce oblong-ovoid bilocular capsules containing numerous radially-symmetrical seeds. *Nuttallanthus canadensis* and *N. texanus* produce flowers with long, slender spurs at the anterior base of the corolla and have been segregated on the basis of flower size and seed coat features; the flowers of *N. canadensis* are relatively small (8 to 11 mm in length, exclusive of the spur) and the surfaces of its seeds possess narrow, longitudinal ridges separating smooth faces, whereas individuals of *N. texanus* produce larger flowers

(14 to 22 mm) and moderately- to densely-tuberculate seeds lacking longitudinal ridges. In both species the flowers are generally greater in length than the subtending pedicels. The inflorescence axes, pedicels and sepals of individuals of *N. texanus* are glabrous, whereas those of *N. canadensis* are sparsely to moderately glandular pubescent.

Nuttallanthus floridanus has traditionally been distinguished from its congeners by the relative length of the pedicels, which greatly exceed the corolla in length, and the small size (5 to 7 mm in length) of the essentially spur-less flowers. In contrast to the straight inflorescence axes of its congeners, those of *N. floridanus* are highly flexuous, with the axes zig-zagging at each flower-bearing node. The inflorescence axes, pedicels and sepals of *N. floridanus* are densely glandular pubescent, and the seeds are characterized by narrow longitudinal ridges separating faces with short, acute ridges and scattered tubercles.

Pennell (1935) proposed that different pollination syndromes explained the variation in floral morphology among species. Our breeding system studies indicate that species of *Nuttallanthus* are primarily autogamous and that pollinators are rarely successful in transferring pollen among individuals (Crawford 2003). The three North American species are cross-incompatible, but the nature of the post-pollination reproductive isolating mechanism was not determined.

A chromosome base number of $x = 6$ has been reported for *Linaria* and for *Nuttallanthus* (Elisens 1986). Previous chromosome counts have been determined for *N. canadensis* and *N. texanus*. Whereas all counts for *N. canadensis* represent the diploid condition (2n = 12; Kapoor et al. 1987), evidence of polyploidy has been reported in *N. texanus*, with meiotic chromosome counts of n = 6 (Ward 1983a, Ward 1983b) and n =

12 (tetraploid; Raven 1963). No chromosome counts have been reported for *N. floridanus*.

While *Nuttallanthus floridanus* has been recognized as a distinct species since its original description as *Linaria floridana* (Chapman 1860), *N. canadensis* and *N. texanus* have been accorded specific status by some authors (Pennell 1935, Sutton 1988) and relegated to varietal rank by others (Pennell 1920, Munz 1926, Rothmaler 1954, Cronquist et al. 1984). These different taxonomic treatments reportedly reflect high levels of variation and intergradation among morphological characters used to distinguish among these taxa (Pennell 1935, Sutton 1988); the intergradation among morphological characters has been attributed to ecological factors and hybridization among species.

This study employed univariate and multivariate analyses of vegetative and reproductive traits measured from field-collected specimens to document the extent and pattern of morphological variation within and among the North American species of *Nuttallanthus*. These analyses were performed subsequent to a study of genetic variation and mating systems in the genus (Crawford 2003). The principal goals of this investigation were to determine the extent of morphological differentiation within and among species in *Nuttallanthus*, to compare the pattern of morphological variation in the genus to hypothesized species boundaries, to determine the degree of correspondence between morphological variation and genetic variation inferred from isozymes, and to evaluate the utility of the morphological characters historically used to delimit these taxa. An additional aim was to determine chromosome numbers for populations of these taxa and to investigate the role of reported differences in chromosome number in the lack of interfertility among these species.

MATERIALS AND METHODS

Morphology. Individual flowering plants were collected from throughout each of the sampled populations and pressed for later examination; where possible, plants possessing both flowers and mature fruits were collected. A total of 242 individuals from 59 populations representing 3 species of *Nuttallanthus* were selected for examination of morphological variation: 113 individuals from 28 populations of *N. canadensis,* 37 individuals from 8 populations of *N. floridanus,* and 92 individuals from 23 populations of *N. texanus* (Table 1; Figure 1). All of the specimens examined were deposited at the Bebb Herbarium of the University of Oklahoma (OKL).

Characters for study were chosen on the basis of prior taxonomic treatments of the *Nuttallanthus* species complex and examination of herbarium specimens and plants in the field. A total of 48 characters was recorded, including 11 vegetative and 37 reproductive traits; of these, 26 were quantitative traits, 14 were ratios of those quantitative traits, two were meristic characters and six were qualitative features (Table 2). Plant height was measured from the top of the root stock to the tip of the tallest fertile stem; stem diameter was measured at the base of the tallest fertile stem. To assess the branching pattern of individual plants, nodal density was defined as the number of leaf-bearing nodes divided by the distance in cm between the base of the longest fertile stem and the first flower-bearing node on that stem. Leaf characters were taken from the largest, fully-expanded leaf present on fertile (aerial) and sterile (basal) stems. Floral characters were obtained from the most recently-opened flower on each individual; bract characters, flowering pedicel length and vestiture density were taken from the bract and pedicel subtending that flower. Fruiting pedicel length was taken from the pedicel

supporting the fruit used for character analysis. To examine the shape of leaves, bracts, floral structures and fruits, length and width measurements of individual structures were combined as ratios. Seeds were collected from an irregular number of mature capsules on each specimen.

Plant height was measured with a ruler; stem diameter, leaf lengths and leaf widths were measured with a vernier caliper. All other quantitative measurements were made using a Wild M5 stereo microscope and an ocular micrometer at 6X and 12X.

Seed characters were observed with a Wild M5 stereo microscope at 25X and 50X. In order to ensure that the seed characters chosen for analysis could be reliably distinguished at these magnifications, seeds from 10 individuals of each of three species of *Nuttallanthus* were examined with the scanning electron microscope. Seeds were obtained from mature capsules collected from field populations or from herbarium specimens (Table 1). Individuals were chosen from throughout the North American range of each species. Representative specimens were affixed to aluminum specimen stubs with double-sided carbon tape and were sputter-coated for 5 minutes with gold/palladium in an Anatech LTD Hummer VI plasma sputterer. These samples were observed with an ETEC Autoscan SEM and photographed with Polaroid 665 film at the Samuel Roberts Noble Electron Microscopy Laboratory at OU. A total of 150 seeds (5 seeds from each of 10 individuals of three *Nuttallanthus* species) was observed with the SEM.

Chromosome counts. Meotic tissues obtained from natural populations and growth chamber-grown plants (Table 1) were used to determine chromosome numbers. A chromosome count was obtained from at least one individual for each population of the

three species represented in the isozyme study (Crawford 2003). Floral buds were fixed in a 3:1:1 solution of 100% ethanol, chloroform and glacial acetic acid for 24 hours and then washed in three changes of 70% ethanol. The fixed floral tissue was incubated in an alcoholic-carmine stain (Snow 1963) for 48 hours at room temperature, then rinsed and stored in 70% ethanol at 12.5°C. Anther primordia were removed from the buds and lightly macerated using needle-point forceps and squashed in 45% acetic acid. Chromosome counts were made and documented photographically with a Leitz Dialux 20 microscope and Delpht Photoautomat 35 mm camera at 1000X using Kodak EliteChrome Tungsten film; all slides are in the possession of the primary author.

Statistical Analyses. Univariate statistical analyses were performed with SPSS for Windows (ver. 11.5.0; SPSS, Inc. 2002). Descriptive statistics and character correlations were calculated for each species; normality tests were performed for each character. Samples departing significantly from normality were log-transformed (continuous variables) or square-root transformed (meristic variables). To compare morphological characters among populations and species, one-way analysis of variance was performed on each character that could be normalized and a one-way Kruskal-Wallis test was used for non-normal samples; post-hoc testing was performed using Fisher's least significant difference test.

Multivariate statistical analyses were performed using SPSS for Windows and NTSYSpc (ver. 2.11c; Rohlf 2002). Ratios were omitted from the multivariate analyses to avoid inordinate weighting of certain characters.

Principal component analysis (PCA) was performed to examine relationships among morphological characters and to evaluate the pattern of morphological variation

among species. Individual specimens served as operational taxonomic units (OTUs). Missing data were excluded from the analyses. Separate PCAs were performed on three data sets: 1) reproductive plus vegetative characters, 2) reproductive characters and 3) vegetative characters. These data sets included both quantitative and meristic characters, so correlation matrices were used in the PCAs. A separate PCA was performed using population means of reproductive and vegetative characters as OTUs in an attempt to detect patterns of geographic variation among populations.

Discriminant functions analysis (DFA) was employed to determine whether species could be reliably distinguished based on the character data. Separate DFAs were performed on three data sets: 1) reproductive plus vegetative characters, 2) reproductive characters and 3) vegetative characters. As DFA is not affected by differences in the scaling of variables (Manly 1994), character data were transformed only if their distributions were non-normal. Missing data were excluded from the analyses.

Dendrograms were generated with the unweighted pair-group method using arithmetical averages (UPGMA). In order to reduce potential distortion resulting from the use of dissimilar scales of measurement in different characters and from differences in the ranges of character values, all continuous variables were log-transformed; the meristic variables were square-root transformed. Population means were calculated for each character data set and matrices of all pair-wise resemblance values among populations were calculated using average taxonomic distance, Euclidean distance and product-moment correlation as coefficients of resemblance. Cophenetic correlations were calculated for each of the dendrograms. The procedures described above were also used to create dendrograms and summary statistics illustrating species-level relationships.

Mantel tests (Mantel 1967) were performed to assess the correlation of average taxonomic distance coefficients calculated from the morphological data sets with a genetic distance matrix based on Nei's 1972 genetic distance coefficient calculated from isozyme data (Crawford 2003). To assess the degree of association of the phenotypic distance between populations within each species and the geographic location of those populations, Spearman's rank-order correlation analysis was perfomed with SPSS for Windows on matrices of average taxonomic distance coefficients and of linear distances (in kilometers) between populations.

RESULTS

Morphological analyses. All of the quantitative and meristic characters assessed in this study varied significantly among the 3 species of *Nuttallanthus* (Table 3). Plants of *Nuttallanthus texanus* are generally more robust than those of their congeners and the measured vegetative and reproductive structures are largest in individuals of this species; the leaf, floral and fruit characters are smallest in *N. floridanus*, and those of *N. canadensis* are intermediate in size. While significant differences in the shapes of these structures (as assessed by combining length and width measurements of structures as ratios) were observed among species, the length and width of leaves of both fertile and sterile stems were correlated with one another. In addition, many of the quantitative measurements of floral structures were correlated, and their correlation coefficients were statistically significant. None of the characters were significantly correlated with latitude or longitude and did not appear to vary clinally within species. Although ranges of some

characters overlapped broadly among species, differences existed in many characters that served to demarcate morphologically-distinct taxonomic groups.

Observation of seeds from each of three species of *Nuttallanthus* with a stereo microscope at 25X and 50X and with the scanning electron microscope indicated that a number of qualitative differences in seed coat morphology existed between species and that many of those differences could be reliably distinguished at the lower magnifications. Seeds of the three species of *Nuttallanthus* were similar in size and color, were four- to seven-angled and radially symmetrical. The seeds of *Nuttallanthus canadensis* were characterized by narrow, entire longitudinal ridges separating interstitial regions with scattered unicellular papillae (Figures 2 through 5). Seeds from individuals of *Nuttallanthus texanus* fit into one of two morphological categories: the seeds of individuals from the southeastern United States, California, Oklahoma and central Texas were essentially terete, exhibited little evidence of longitudinal ridges and possessed faces densely covered with acute, multicellular tubercles (Figures 6 and 7); the seeds of individuals from Arkansas, Louisiana, eastern Texas and one population in central Oklahoma were distinctly angular and possessed faces with low, rounded ridges and scattered multicellular tubercles (Figures 8 and 9). Most of the populations of *N. texanus* examined in this study produced only one of these seed types; populations of *N. texanus* producing different types of seeds were also distinguishable isozymically (Crawford 2003). One population in central Oklahoma (population 58) was composed of individuals belonging to both of these groups of *N. texanus*; that population was divided into two sub-populations (58a and 58b) for analysis. No unicellular papillae were observed on any specimen of *N. texanus*, but these features were too small to be

distinguished with a dissecting microscope. The seeds of *Nuttallanthus floridanus* were characterized by narrow, entire longitudinal ridges separating faces with short, acute ridges and scattered multicellular tubercles and unicellular papillae (Figures 10 and 11).

Principal component analysis (PCA) of reproductive and vegetative characters clearly separated individuals of the three species in scatterplots (Figure 12); the first three principal components accounted for 69.1% of the variance in the data set. The first principal component explained 48.0% of that variation, consisting primarily of information from characters related to floral bract, calyx, corolla and fruit size; all of these characters possessed positive eigenvectors, indicating that the first principal component is largely an indicator of the size of reproductive structures (Appendix 1). A high negative loading was observed for pedicel vestiture density, and the presence or absence of acute longitudinal ridges on seed surfaces also contributed substantially. The second principal component accounted for an additional 12.2% of the variance in the data set and possessed high positive loadings for the pedicel length of flowers and fruits and for corolla color; the remaining two seed coat characters (presence or absence of obtuse sinuate ridges and multicellular tubercles on seed surfaces) contributed to this component, as did the shape of the inflorescence axis. The third principal component explained 8.89% of the total variance and gave its highest loadings for vegetative characters. The first five principal components possessed eigenvalues greater than 1; considering only those principal components (after Legendre et al. 1998, Kachigan 1991) accounted for only 77.1% of the total variation in the data set. This suggested that some of the assessed characters varied independently of others and that morphological variation in these species cannot be reduced to a few orthogonal principal component

axes. However, PCA of reproductive and vegetative characters clearly distinguished three morphologically distinct groups. Similar results were obtained in PCA of reproductive characters only, with the first three components accounting for 79.8% of the variance in the data set (Figure 13). The analysis of vegetative characters did not clearly separate individuals in a few dimensions; a large degree of overlap was observed among species (Figure 14). The PCA using population means of reproductive and vegetative characters as OTUs clearly separated populations of the three species in scatterplots (Figure 15); the first and second principal components accounted for 55.5% and 15.1%, respectively, of the variance in the data set. The third principal component explained only 7.8% of the total variance, but plotting the results in three dimensions illustrated the pronounced phenotypic differentiation among the three species of *Nuttallanthus* and distinguished two principal groups of *N. texanus* (Figure 16).

Discriminant function analysis (DFA) of morphological characters indicated that individual plants may be assigned to their respective species with a high degree of accuracy: 100% of individuals were correctly assigned on the basis of all characters and reproductive characters alone (Table 4). DFA of the quantitative and meristic characters in these data sets (omitting qualitative characters) yielded similar results, with a minimum of 98.6% of individuals correctly assigned to species. DFA of vegetative characters alone was less accurate, with predicted group memberships ranging from 71.9% to 85.3% (Table 4).

Dendrograms summarizing the phenotypic similarities among populations and species of *Nuttallanthus* were produced using UPGMA (Sneath et al. 1973) in combination with average taxonomic distance, Euclidean distance and product-moment

correlation coefficients based on reproductive and vegetative characters. Regardless of the resemblance coefficient employed, the specimens examined fell into three discrete groups with conspecific populations clustering together. A matrix of average taxonomic distance values resulted in the highest cophenetic correlation among the dendrograms produced (0.925); consequently, that dendrogram is reproduced here (Figure 17). Two discrete clusters of populations of *Nuttallanthus texanus* are apparent; one cluster is predominately composed of populations from the southeastern United States, California, Oklahoma and central Texas, whereas populations from Arkansas, Louisiana, eastern Texas and Oklahoma comprise the second cluster.

The results of the morphological analyses described above were broadly consistent with those of an analysis of genetic variation in the North American species of *Nuttallanthus* (Crawford 2003). Mantel tests revealed a significant correlation between average taxonomic distance coefficients calculated from the reproductive and vegetative character data set and Nei's (1972) genetic distance coefficients calculated from isozyme data ($r = 0.749$; $p = 0.002$), as well as between reproductive characters alone and genetic distance ($r = 0.810$; $p = 0.002$) and between genetic distance and vegetative characters alone ($r = 0.164$; $p = 0.007$). Spearman's rank-order correlation analysis was performed with SPSS for Windows on matrices of average taxonomic distance coefficients and of linear distances (in kilometers) between populations. These analyses indicated positive and statistically-significant associations between phenotypic distance and linear distance between populations for each species: *Nuttallanthus canadensis* ($r_s = 0.372$, $p < 0.001$), *N. floridanus* ($r_s = 0.493$, $p = 0.008$) and *N. texanus* ($r_s = 0.246$, $p = 0.001$).

Chromosome counts. Meiotic tissues were used to obtain chromosome counts from at least one individual for each population of the three species represented in the isozyme study (Crawford 2003). All counts were n = 6.

DISCUSSION

Univariate and multivariate analyses of morphometric data measured from field-collected specimens revealed significant morphological gaps between three principal groups of North American *Nuttallanthus*. These results were consistent with the recent treatment of the genus by Sutton (1988) and supported the recognition of three species in North America: *Nuttallanthus canadensis*, *N. floridanus* and *N. texanus*. In addition, the analyses affirmed the utility of the morphological characters that have been used to distinguish among these taxa; although the ranges of some of the measured characters overlapped among taxa, all of the quantitative and meristic characters assessed in this study varied significantly among the 3 species of *Nuttallanthus* (Table2; Table 3). None of the characters were significantly correlated with latitude or longitude and did not appear to vary clinally within species but rather demarcated morphologically-distinct groups of populations. Discriminant function analysis (DFA) employing all measured characters associated 100% of individual plants with their respective species, and principal component analysis (PCA) demonstrated that many reproductive and vegetative traits contributed to the observed morphological differentiation among taxa. While both vegetative and reproductive characters varied significantly among taxa and provided high loadings on principal component axes, reproductive characters provided much better discrimination than did vegetative characters.

Species of *Nuttallanthus* may be readily identified on the basis of seed coat exomorphology, corolla, spur and pedicel length and inflorescence axis shape, characters traditionally used to discriminate among these taxa and in which little intergradation among species was observed by the primary author. These results were consistent with those of isozyme and breeding system analyses in the North American species of *Nuttallanthus* (Crawford 2003), which demonstrated a high degree of genetic differentiation and reproductive isolation among these species. In addition, a pronounced geographic component in the patterns of genetic and morphological differentiation among populations of these species was evident in the data sets; conspecific populations that were geographically distant exhibited greater genetic and morphological divergence relative to geographically-proximate populations, suggesting decreasing levels of gene flow with increased physical distance among populations. Genetic and morphological similarities among conspecific populations were statistically correlated with each other and with geographic distance across the sampled range of each species of *Nuttallanthus*; such congruence in genetic and morphological differentiation generally results from relatively long periods of divergent evolution among isolated populations (Crawford 1989). Although these species are currently sympatric in a portion of their ranges, the observed divergence in numerous morphological characters and the accumulation of unique alleles and substantial allele frequency differences among species of *Nuttallanthus* are consistent with a geographic (allopatric) mode of speciation, in which extrinsic barriers to gene flow lead to a gradual divergence among isolated sets of populations (Crawford 1990; Grant 1981). No evidence of interspecific hybridization, polyploidy or recent divergence among these species was apparent in the data; while phylogenetic

relationships among species of *Nuttallanthus* are unclear (due to similar genetic divergence values among species), no indication of progenitor-derivative relationships among these species was evident (Crawford 2003). The observed intersterility among species of *Nuttallanthus* was not due to differences in chromosome number, as all of the populations represented in this study yielded a meiotic count of n = 6. A pattern of morphological, genetic and reproductive differentiation similar to that observed in *Nuttallanthus* has been reported in a number of plant genera in which geographical speciation has been inferred (Elisens et al. 1988; Vanderpool et al. 1991; Warwick et al. 1985). Estimated divergence times among species of *Nuttallanthus* based on Nei's (1987) stepwise mutation rate models (I_E and I_{EA}) calculated from genetic identities indicate that these species diverged in the late Miocene and Pliocene (Crawford 2003). During portions of the Pliocene, the east Gulf Coastal Plain, the current center of taxonomic diversity of *Nuttallanthus*, consisted of a number of isolated fragments (Walker et al. 1987). These fragmented areas of the coastal plain apparently served as refugia for a number of plant species (Estill et al. 2001; Sorrie et al. 2001) and may have provided opportunities for speciation among geographically-isolated progenitor populations of *Nuttallanthus*.

Significant levels of character differentiation were also detected among two groups of populations of *N. texanus* (Figure 17). These two groups differed significantly in 17 of the 42 quantitative and meristic characters assessed in this study. Individuals of group 2 tended to be more robust (differing from group 1 in height, stem diameter and nodal density) and produced larger flowers, which differed from those of group 1 in corolla length, tube diameter and spur length. A Mantel test revealed no correlation

between average taxonomic distance coefficients calculated from the reproductive character data set and the vegetative character data set ($r = 0.056$; $p = 0.137$), suggesting that the observed differences in floral size were not the result of differences in overall plant size. DFA indicated that individual plants of *N. texanus* may be assigned to their group of origin with a high degree of accuracy: 98.2% of individuals of group 1 and 100% of individuals of group 2 were correctly assigned on the basis of all characters. DFA of the quantitative and meristic characters in these data sets (omitting qualitative characters) yielded similar results, with a minimum of 96.4% of individuals correctly assigned. Whereas most of the assessed differences between groups 1 and 2 in vegetative and reproductive characters were quantitative in nature, individuals from these groups differed qualitatively in seed exomorphology (Figures 6 - 9). Some authors have suggested that relatively robust plants with seeds similar to those of group 2 may be attributed to hybridization between *Nuttallanthus canadensis* and *N. texanus* (Sutton 1988), but the results of our morphological, isozyme and breeding system studies provide no evidence of a hybrid origin of group 2. All individuals from these two groups shared a marker allele (6PGD-2a) not found in *N. canadensis* or *N. floridanus*, and although members of both groups exhibited intersterility with individuals of *N. canadensis* and *N. floridanus*, no reduction in interfertility was observed between the two groups (Crawford 2003). Considering the minor degree of morphological and genetic differentiation among these groups (relative to the extent of divergence among species of *Nuttallanthus*) the recognition of infraspecific taxa in *N. texanus* does not seem warranted.

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Table 1. Numbered collection data for 60 populations representing three species of *Nuttallanthus* examined for morphological and chromosome number variation. Population codes are given in bold numbers; OTUs included in population-level morphological analyses are indicated by an asterisk (*). Collection numbers for specimens providing seed for environmental chamber-grown plants used for chromosome number determination are indicated by a plus sign (+). Voucher collections are deposited at OKL unless otherwise indicated.

Nuttallanthus canadensis

Alabama: Baldwin Co.: **1*** = 30.414N 87.598W, 0.1 mi. NE of Highway 98 & S Michigan, Elberta, *Crawford 353, 354, 355, 356, 357, 359*+; **2** = 30.238N 87.882W, Miller Memorial Cemetery, Miller Memorial Road and Highway 180 intersection, *Crawford 684, 685*. Mobile Co.: **3** = 30.433N 88.144W, grounds of Bellingrath Gardens Estate, *Crawford 321, 322, 323*; **4** = 30.243N 88.078, E end of Dauphin island in seaside dunes, *Crawford 649*, *Crawford 650*+.

Delaware: Sussex Co.: **5*** = 38.574N 75.056W, 2.0 mi. N of the Highway 1 & Highway 26 intersection, *Crawford 203, 204*+*, 205, 206, 207, 208, 209, 210.*

Florida: Bay Co.: **6*** = 30.204N 85.847W, 3.0 mi. W of the Highway Alt98 & Highway 392 intersection, *Crawford 375, 376, 377, 378, 379*+*, 710, 711, 712, 713*; Calhoun Co.: **7** = 30.464N 85.045W, 1.0 mi. N of Blountstown, *Crawford 852*. Franklin Co.: **8*** = 29.833N 84.876W, 8.6 mi. N of the Highway 65 & Highway 98 intersection, *Crawford 400, 401*+*, 402, 403, 404, 405*; **9*** = 29.909N 84.394W, dunes on Lighthouse Point, W end of peninsula, *Crawford 444, 445*+*, 446, 447, 448*; **10** = 29.853N 84.664W, open pine woodland near Carrabelle, N of 3rd Street, *Crawford 807*. Lafayette Co.: **11*** = 30.139N 83.290W, 0.1 mi. N of Highway 27, 8.8 mi. W of Mayo, *Crawford 490, 491, 492*+. Wakulla Co.: **12*** = 30.136N 84.326W, 0.1 mi. NE of the Highway 98 & Spring Creek Highway intersection, *Crawford 812, 813, 814, 815, 816*+. Walton Co.: **13** =

Table 1 cont.

30.309N 86.102W, shore of Eastern Lake, E of Seagrove Beach, *Crawford 703, 704, 705.*

Georgia: Camden Co.: **14*** = 30.759N 81.658W, meadow above N bank of St. Mary's River, 0.1 mi. E of Interstate 95, *Crawford 503, 504, 505, 506, 507, 508, 511*$^{+}$. Candler Co.: **15*** = 32.355N 81.989W, 11.7 mi. SE of the Interstate 16 & Highway 57 intersection, *Crawford 592, 593, 594, 595, 601*$^{+}$. Glynn Co.: **16*** = 31.020N 81.435W, meadow near St. Andrew picnic area, S Riverside Drive, Jekyll Island, *Crawford 529, 530, 531, 532*$^{+}$. Liberty Co.: **17*** = 31.675N 81.414W, edge of Beltowne marsh 3.6 mi. S of Retreat, *Crawford 542, 543, 544, 545*$^{+}$.

Maryland: Caroline Co.: **18*** = 38.817N 75.748W, 1.5 mi. E of the Highway 404 & Highway 16 intersection, *Crawford 241, 242, 243, 244*$^{+}$. Worcester Co.: **19*** = 38.096N 75.499W, fallow cornfield E of Highway 113 and 0.3 mi. S of mile 6 marker, *Crawford 180*$^{+}$*, 181, 182, 183, 184, 185.*

Massachusetts: Middlesex Co.: **20*** = 42.504N 71.265W, sandy roadside at edge of pine woodland, Concord Field Station, Bedford, *Crawford 898, 899*$^{+}$*, 900.*

North Carolina: Currituck Co.: **21*** = 36.278N 75.915W, 0.5 mi. N of the Highway 158 & Highway 3E intersection, *Crawford 115, 116, 117, 119*$^{+}$. Dare Co.: **22*** = 35.261N 75.579W, Hatteras Island, near intersection of Highway 12 & Paradise Lane, *Crawford 101, 102, 103, 104*$^{+}$. Duplin Co.: **23*** = 34.926N 77.652W, S of Highway 24 and 1 mi. W of Duplin/Onslow county line, *Crawford 88, 89, 90*$^{+}$. Hoke Co.: **24*** = 35.007N 79.305W, SW of the Highway 211 & SR1202 intersection, *Crawford 62, 63, 64, 65, 66, 67, 68*$^{+}$.

South Carolina: Beaufort Co.: **25*** = 32.377N 80.832W, meadow on W bank of Chechessee R, 0.1 mi. SE of Highway 170 bridge, *Crawford 569, 570, 571, 572, 576*$^{+}$.

Table 1 cont.

Virginia: Accomack Co.: **26*** = 37.912N 75.356W, Chincoteague Island, S of Highway 175, near causeway to Assateague Island, *Crawford 154, 155, 156, 157, 158, 159, 170*[+]. Northampton Co.: **27*** = 37.145N 75.967W, 0.4 mi. S of the Highway 13 & Latimer Siding Road intersection, *Crawford 128, 129*[+]*, 130, 131, 132, 133.* Orange Co.: **28*** = 38.261N 77.980W, S of Highway 20, near the Highway 20 & Village Road intersection, *Crawford 254, 255, 256, 258*[+].

Nuttallanthus floridanus

Alabama: Baldwin Co.: **29*** = 30.238N 87.882W, Miller Memorial Cemetery, Miller Memorial Road and Highway 180 intersection, *Crawford 668, 669, 670, 671, 672, 678*[+]. Mobile Co.: **30*** = 30.243N 88.078, E end of Dauphin island in seaside dunes, *Crawford 652, 653, 654, 655, 656, 657*[+].

Florida: Bay Co.: **31*** = 30.204N 85.847W, S of Beach Front Road, 3.0 mi. W of the Highway Alt98 & Highway 392 intersection, *Crawford 719, 720, 721*[+]*, 722.* Franklin Co.: **32*** = 29.909N 84.394W, dunes on Lighthouse Point, W of Alligator Point village, W end of peninsula, *Crawford 462*[+]*, 463, 464, 465, 466*; **33*** = 29.723N 84.890W, 0.2 mi. S of the Highway 98 & Highway 300 intersection, *Crawford 748, 749*[+]*, 750, 751, 752*; **34*** = 29.724N 84.899W, sandy meadow SE of E end of Gorrie Bridge, E bank of Apalachicola River, *Crawford 767, 768, 769, 770, 771*[+]; **35*** = 29.853N 84.664W, N of 3rd Street in Carrabelle, *Crawford 792, 793, 794, 795*[+]*, 796.* Marion County: Rt. 44, 1 mile N of the intersection of Rt. 44 and Rt. 40, W of Astor. Godfrey 80417 (FSU). Putnam Co.: **36*** = 29.623N 81.912W, 0.1 mi. S of Highway 20, W of Interlachen, FL, *Crawford 832*[+]*, 833, 834, 835, 836.*

Table 1 cont.

Nuttallanthus texanus

Alabama: Baldwin Co.: **37*** = 30.414N 87.598W, 0.1 mi. NE of Highway 98 & S Michigan, Elberta, *Crawford 334, 335, 336, 337, 338, 342*$^{+}$. Mobile Co.: **38*** = 30.243N 88.078, E end of Dauphin island in seaside dunes, *Crawford 636, 637, 638, 639*$^{+}$.

Arkansas: Conway Co.: **39*** = 35.171N 92.755W, 0.1 mi. S of Interstate 40 mile marker 107, *Crawford 49, 50, 51, 52*$^{+}$. Crawford Co.: **40*** = 35.528N 94.041W, 0.25 mi. S of Interstate 40, 1.0 mi. W of Mulberry exit, *Crawford 36*$^{+}$*, 37, 38*; Logan Co.: **41** = 35.300N 93.634W, Subiaco Academy grounds, Subiaco, *Crawford 267, 268, 269, 274*$^{+}$.

California: Monterrey Co.: **42*** = 36.511N 121.942W, grown from seed collected at Point Lobos State Reserve, *Crawford 917, 918, 919, WJ Elisens 901*$^{+}$. Santa Barbara Co.: **43*** = 34.044N 119.718W, grown from seed collected at Pelican Bay, Santa Cruz Island, *Crawford 926, 927, 928, WJ Elisens 900*$^{+}$.

Florida: Bay Co.: **44** = 30.204N 85.847W, 3.0 mi. W of the Highway Alt98 & Highway 392 intersection, *Crawford 374*. Calhoun Co.: **45** = 30.464N 85.045W, 1.0 mi. N of Blountstown, *Crawford 861*. Franklin Co.: **47*** = 29.909N 84.394W, dunes on Lighthouse Point, W end of peninsula, *Crawford 426, 427, 428, 429, 430, 431*$^{+}$; **48** = 29.723N 84.890W, 0.2 mi. S of the Highway 98 & Highway 300 intersection, *Crawford 761*; Wakulla Co.: **50*** = 30.136N 84.326W, 0.1 mi. NE of the Highway 98 & Spring Creek Highway intersection, *Crawford 477, 478, 479, Crawford 827, 828, Crawford 830*$^{+}$. Walton Co.: **51*** = 30.309N 86.102W, shore of Eastern Lake, E of Seagrove Beach, *Crawford 691, 692*$^{+}$*, 693, 694, 695.*

Georgia: Candler Co.: **52*** = 32.355N 81.989W, meadow 11.7 mi. SE of the Interstate 16 &

Table 1 cont.

Highway 57 intersection, *Crawford 583, 584, 585, 586, 587*$^{+}$.

Louisiana: St. Landry Parish: **53*** = 30.540N 92.028W, 0.2 mi. E of the Interstate 49 & Highway 190 intersection, *Crawford 293, 294, 295*$^{+}$. St. Tammany Parish: **54*** = 30.298N 89.666W, 0.3 mi. S of Interstate 12, high on W bank of Pearl River, *Crawford 306*$^{+}$*, 307, 308.*

North Carolina: Currituck Co.: **55** = 36.278N 75.915W, 0.5 mi. N of the Highway 158 & Highway 3E intersection, *PT Crawford 114.*

Oklahoma: Cleveland Co.: **56*** = 35.214N 97.328W, T9N R1W Sec 32 NW 1/4, *Crawford 31, 32, 33, 34, 35, 975, 976, 977*, floral buds collected 24 April 2000. Garvin Co.: **57*** = 34.708N 97.543W, T3N R3W Sec 30 NE 1/4, *Crawford 26, 27, 28, 29, 30, 972, 973, 974*, floral buds collected 23 April 2000; **58*** = 34.745N 97.548W, T3N R3W Sec 19 SE 1/4, *Crawford 934, 935, 936, 937, 938, 939, 940, 941, 942, 943*, floral buds collected 23 April 2000. Johnston Co.: **59*** = 34.327N 96.770W, T3S R5E Sec 3 NE 1/4, "Islands" of shallow soil in Tishomingo granite of Ten-acre Rock, *Crawford 886, 887, 969, 970, 971*, floral buds collected 22 April 2000.

South Carolina: Beaufort Co.: **60*** = 32.377N 80.832W, meadow on W bank of Chechessee R, 0.1 mi. SE of Highway 170 bridge, *Crawford 555, 556, 557, 558, 560*$^{+}$.

Texas: Harrison Co.: **61*** = 34.470N 94.595W, 0.1 mi. N of Interstate 20, 0.8 mi. W of exit 604, *Crawford 280, 281, 282, 288*$^{+}$. Smith Co.: **62*** = 32.469N 95.389W, 0.25 mi. S of Interstate 20 near mile marker 557, *Crawford 866, 867, 868*$^{+}$*, 869, 870.*

Table 2. Description of 26 quantitative characters, 14 ratios of quantitative characters, 2 meristic characters and 6 qualitative characters included in a morphometric analysis of three *Nuttallanthus* species. Qualitative character state codings are given in brackets.

Vegetative characters:

1. Plant height (top of the root stock to tallest fertile stem tip)
2. Stem diameter (at base of tallest fertile stem)
3. Number of fertile stems present
4. Number of sterile stems present
5. Nodal density (number of leaf-bearing nodes divided by distance in cm between base of longest fertile stem and first flower-bearing node on that stem)
6. Sterile stem leaf length (base to tip of blade)
7. Sterile stem leaf width (maximum width)
8. Sterile stem leaf length/width ratio
9. Fertile stem leaf length (base to tip of blade)
10. Fertile stem leaf width (maximum width)
11. Fertile stem leaf length/width ratio

Reproductive characters:

12. Inflorescence axis: straight [1], flexuous [2]
13. Floral bract length (bract subtending the flower used for character analysis)
14. Floral bract width (bract subtending the flower used for character analysis)
15. Floral bract length/width ratio
16. Flowering pedicel length (pedicel supporting the flower used for character analysis)
17. Fruiting pedicel length (pedicel supporting the fruit used for character analysis)

Table 2 cont.

18. Pedicel vestiture density: essentially glabrous [1], sparsely pubescent [2], densely pubescent [3]
19. Adaxial calyx lobe length
20. Adaxial calyx lobe width at base
21. Adaxial calyx lobe length/width ratio
22. Abaxial calyx lobe length
23. Abaxial calyx lobe width at base
24. Abaxial calyx lobe length/width ratio
25. Corolla color (distribution and color of pigmentation): blue with white palate [1], pink with white palate [2], lavender throughout [3], dark purple throughout [4]
26. Corolla length (tip of abaxial lobe to base of ovary)
27. Adaxial corolla lobe length
28. Adaxial corolla lobe width
29. Adaxial corolla lobe length/width ratio
30. Adaxial corolla sinus depth
31. Adaxial corolla lobe length/sinus depth ratio
32. Abaxial corolla lobe sinus depth
33. Abaxial central corolla lobe width
34. Abaxial corolla sinus depth/lobe width ratio
35. Corolla tube length (base to mouth of tube)
36. Corolla tube diameter (mouth of tube)
37. Corolla tube length/diameter ratio
38. Corolla length/corolla tube length ratio

Table 2 cont.

39. Corolla spur length

40. Corolla spur diameter at base

41. Corolla spur length/diameter ratio

42. Corolla spur length/corolla length ratio

43. Capsule length (largest capsule)

44. Capsule diameter (at maximum)

45. Capsule length/diameter ratio

46. Acute, entire longitudinal ridges on seed surface: present [1], absent [2]

47. Low, obtuse, sinuate ridges on seed surface: present [1], absent [2]

48. Acute, discrete multicellular tubercles on seed surface: present [1], absent [2]

Table 3. Descriptive statistics for 42 quantitative and meristic morphological characters utilized in morphometric analyses of three species of *Nuttallanthus*. Parentheses denote the number of populations (n) and the number of specimens (N) examined. Means and standard deviations are provided, as are the results of ANOVA, Kruskal-Wallis and a posteriori tests, with species ordered (left to right) from low to high values. Lines above and below species abbreviations connect species that do not differ significantly for a given variable. Dimensions are in mm unless otherwise noted.

Character	*N. canadensis* (n = 28, N = 113) Mean	SD	*N. floridanus* (n = 8, N = 37) Mean	SD	*N. texanus* (n = 23, N = 92) Mean	SD	ANOVA	a posteriori test
Vegetative:								
1. Height (cm)	38.9	7.37	29.4	8.04	40.7	8.67	$F = 27.7$; $p < 0.001$	*flor* *can* *tex*
2. Stem width	1.48	0.35	1.17	0.41	1.64	0.40	$F = 20.8$; $p < 0.001$	*flor* *can* *tex*
3. Fertile stem number	1.88	1.33	2.57	2.17	1.53	0.94	$X^2 = 9.05$; $p = 0.001$	*tex* *can* *flor*
4. Sterile stem number	9.25	5.90	13.0	12.0	8.91	6.27	$F = 4.64$; $p = 0.011$	*tex* *can* *flor*
5. Nodal density	0.61	0.16	0.76	0.19	0.75	0.18	$F = 20.5$; $p < 0.001$	*tex* *flor* *can*
6. Sterile leaf length	4.96	1.65	3.83	0.94	6.15	2.07	$F = 23.7$; $p < 0.001$	*flor* *can* *tex*
7. Sterile leaf width	1.79	0.48	1.68	0.55	2.23	0.63	$F = 19.5$; $p < 0.001$	*flor* *can* *tex*
8. Sterile leaf length/width	2.96	1.47	2.39	0.59	2.79	0.70	$F = 3.83$; $p = 0.023$	*flor* *tex* *can*

Table 3 cont.

Character	*N. canadensis* (n = 28, N = 113) Mean	SD	*N. floridanus* (n = 8, N = 37) Mean	SD	*N. texanus* (n = 23, N = 92) Mean	SD	ANOVA	a posteriori test
9. Fertile leaf length	20.8	6.19	18.7	6.25	23.0	4.93	$F = 8.10$; $p < 0.001$	*flor can tex*
10. Fertile leaf width	1.25	0.29	0.81	0.20	1.86	0.57	$F = 104$; $p < 0.001$	*flor can tex*
11. Fertile leaf length/width	16.9	4.60	23.1	7.72	12.9	2.73	$F = 64.9$;$p < 0.001$	*tex can flor*
Reproductive:								
13. Floral bract length	2.04	0.34	1.59	0.43	2.65	0.49	$F = 100$; $p < 0.001$	*flor can tex*
14. Floral bract width	0.62	0.10	0.48	0.09	0.96	0.19	$X^2 = 165$; $p < 0.001$	*flor can tex*
15. Floral bract length/width	3.36	0.61	3.32	0.70	2.81	0.48	$F = 24.5$; $p < 0.001$	*tex flor can*
16. Pedicel length (flower)	2.40	0.68	5.46	0.97	3.60	1.07	$F = 154$; $p < 0.001$	*can tex flor*
17. Pedicel length (fruit)	3.64	0.92	10.2	1.64	5.25	1.23	$F = 414$; $p < 0.001$	*can tex flor*
19. Adaxial calyx lobe length	2.25	0.25	1.97	0.25	2.78	0.42	$F = 107$; $p < 0.001$	*flor can tex*
20. Adaxial calyx lobe width	0.57	0.10	0.52	0.10	0.79	0.16	$F = 99.7$; $p < 0.001$	*flor can tex*
21. Adaxial lobe length/width	4.01	0.65	3.82	0.56	3.60	0.59	$F = 11.4$; $p < 0.001$	*tex flor can*
22. Abaxial calyx lobe length	2.63	0.30	2.17	0.29	3.30	0.42	$F = 172$; $p < 0.001$	*flor can tex*

Table 3 cont.

Character	*N. canadensis* (n = 28, N = 113) Mean	SD	*N. floridanus* (n = 8, N = 37) Mean	SD	*N. texanus* (n = 23, N = 92) Mean	SD	ANOVA	a posteriori test
23. Abaxial calyx lobe width	0.83	0.10	0.69	0.10	1.22	0.15	F = 359; p < 0.001	*flor can tex*
24. Abaxial lobe length/width	3.19	0.39	3.18	0.44	2.74	0.39	F = 36.2; p < 0.001	*tex flor can*
26. Corolla length	8.75	1.31	6.83	1.08	12.3	1.82	F = 227; p < 0.001	*flor can tex*
27. Adaxial corolla lobe length	3.20	0.56	2.37	0.51	4.67	0.74	F = 229; p < 0.001	*flor can tex*
28. Adaxial corolla lobe width	1.77	0.41	1.22	0.35	2.07	0.51	F = 49.2; p < 0.001	*flor can tex*
29. Adaxial lobe length/width	1.87	0.35	1.99	0.30	2.31	0.39	F = 38.3; p < 0.001	*can flor tex*
30. Adaxial corolla lobe sinus depth	1.93	0.53	0.95	0.40	2.34	0.68	F = 78.2; p < 0.001	*flor can tex*
31. Adaxial lobe length/sinus depth	1.74	0.36	2.81	1.03	2.12	0.57	F = 55.4; p < 0.001	*can tex flor*
32. Abaxial corolla lobe sinus depth	2.62	0.64	1.93	0.39	3.81	0.78	F = 132; p < 0.001	*flor can tex*
33. Abaxial corolla lobe width	2.81	0.74	1.70	0.54	4.01	0.93	F = 126; p < 0.001	*flor can tex*
34. Abaxial lobe sinus depth/width	0.96	0.19	1.18	0.22	0.96	0.17	F = 22.3; p < 0.001	*can tex flor*
35. Corolla tube length	3.17	0.41	2.49	0.39	3.71	0.40	F = 125; p < 0.001	*flor can tex*
36. Corolla tube diameter at mouth	1.16	0.16	0.96	0.17	1.51	0.23	F = 138; p < 0.001	*flor can tex*

Table 3 cont.

Character	*N. canadensis* (n = 28, N = 113) Mean	SD	*N. floridanus* (n = 8, N = 37) Mean	SD	*N. texanus* (n = 23, N = 92) Mean	SD	ANOVA	a posteriori test
37. Corolla tube length/diameter	2.78	0.42	2.64	0.41	2.50	0.40	$F = 11.5$; $p < 0.001$	*tex flor can*
38. Corolla length/tube length	2.77	0.30	2.77	0.36	3.32	0.44	$F = 62.2$; $p < 0.001$	*can flor tex*
39. Corolla spur length	4.38	1.28	0.26	0.18	7.40	1.55	$F = 417$; $p < 0.001$	*flor can tex*
40. Corolla spur diameter at base	0.53	0.09	0.30	0.14	0.71	0.14	$F = 162$; $p < 0.001$	*flor can tex*
41. Corolla spur length/diameter	8.37	2.27	0.83	0.32	10.5	2.44	$F = 241$; $p < 0.001$	*flor can tex*
42. Corolla length/spur length	2.18	0.85	32.4	19.3	1.77	0.78	$F = 968$; $p < 0.001$	*tex can flor*
43. Capsule length	3.26	0.32	2.66	0.32	3.88	0.45	$F = 147$; $p < 0.001$	*flor can tex*
44. Capsule width	2.96	0.31	2.36	0.28	3.38	0.30	$F = 146$; $p < 0.001$	*flor can tex*
45. Capsule length/width	1.10	0.06	1.13	0.09	1.15	0.11	$F = 6.98$; $p = 0.001$	*can flor tex*

Table 4. Predicted group memberships (as percentages) of individuals of three species of *Nuttallanthus* based on Discriminant Function Analysis.
N = Number of sampled individuals / species.

A. Vegetative and reproductive characters

Species	N	*N. canadensis*	*N. floridanus*	*N. texanus*
N. canadensis	76	**100.0**	0	0
N. floridanus	32	0	**100.0**	0
N. texanus	71	0	0	**100.0**

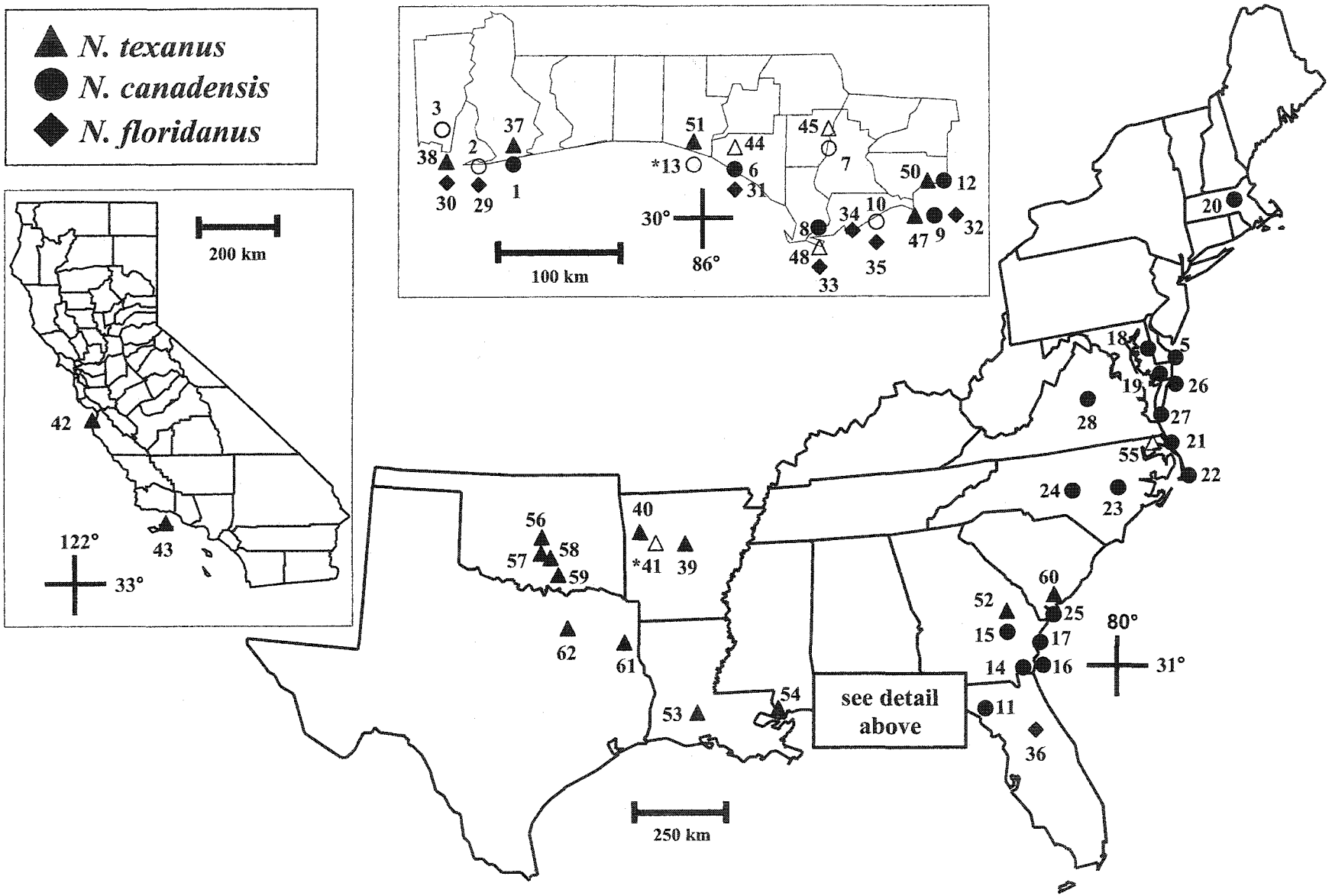
B. Reproductive characters

Species	N	*N. canadensis*	*N. floridanus*	*N. texanus*
N. canadensis	87	**100.0**	0	0
N. floridanus	37	0	**100.0**	0
N. texanus	73	0	0	**100.0**

C. Vegetative characters

Species	N	*N. canadensis*	*N. floridanus*	*N. texanus*
N. canadensis	102	**85.3**	4.9	9.8
N. floridanus	32	28.1	**71.9**	0
N. texanus	89	23.6	0	**76.4**

Figure 1. Numbered collection localities for 59 populations representing three species of *Nuttallanthus* examined for morphological and chromosome number variation. Collection data are listed in Table 1. Filled symbols (▲●◆) refer to populations included in population-level morphological analyses; hollow symbols (△○◇) refer to populations only included in species-level morphological analyses. Population 58 was divided into two subpopulations (58a and 58b) for analysis. Chromosome counts were obtained for all populations represented by filled symbols and for the two populations represented by hollow symbols and indicated by an * (Table 1).


N. texanus
N. canadensis
N. floridanus
200 km
122°
33°
100 km
30°
86°
250 km
80°
31°
see detail above

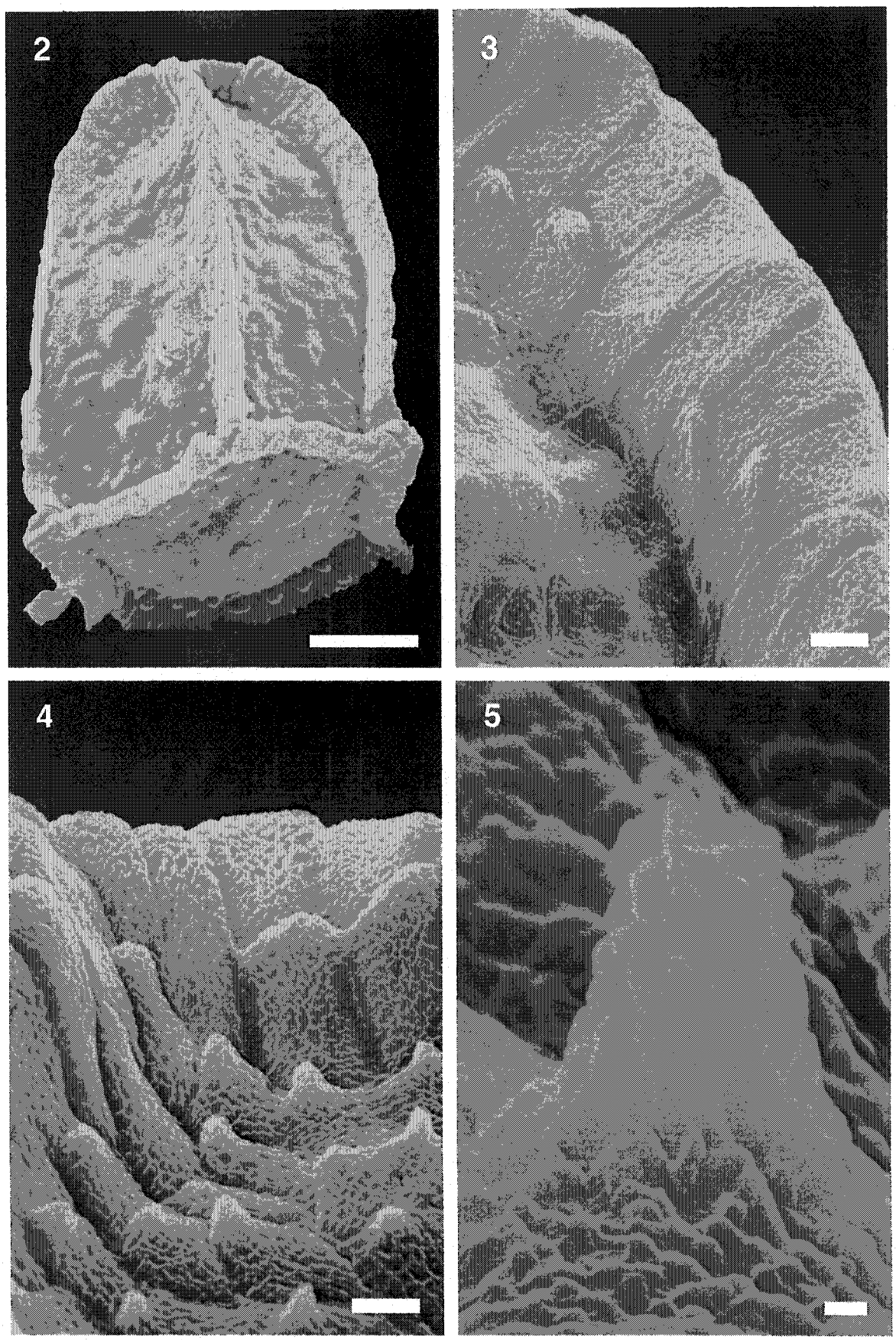
Figures 2 - 5: Scanning electron micrographs of seeds of *Nuttallanthus canadensis*.

Figure 2. Oblique lateral view of an angular seed, hilum to top (Crawford 17). Scale = 100 μ m.

Figure 3: Expanded view of longitudinal ridge and face (Crawford 17). Scale = 10 μ m.

Figure 4: Oblique view of distal end of seed (Crawford 19). Scale = 10 μ m.

Figure 5: Oblique lateral view of a unicellular papilla (Crawford 19). Scale = 1 μ m.


2
3
4
5

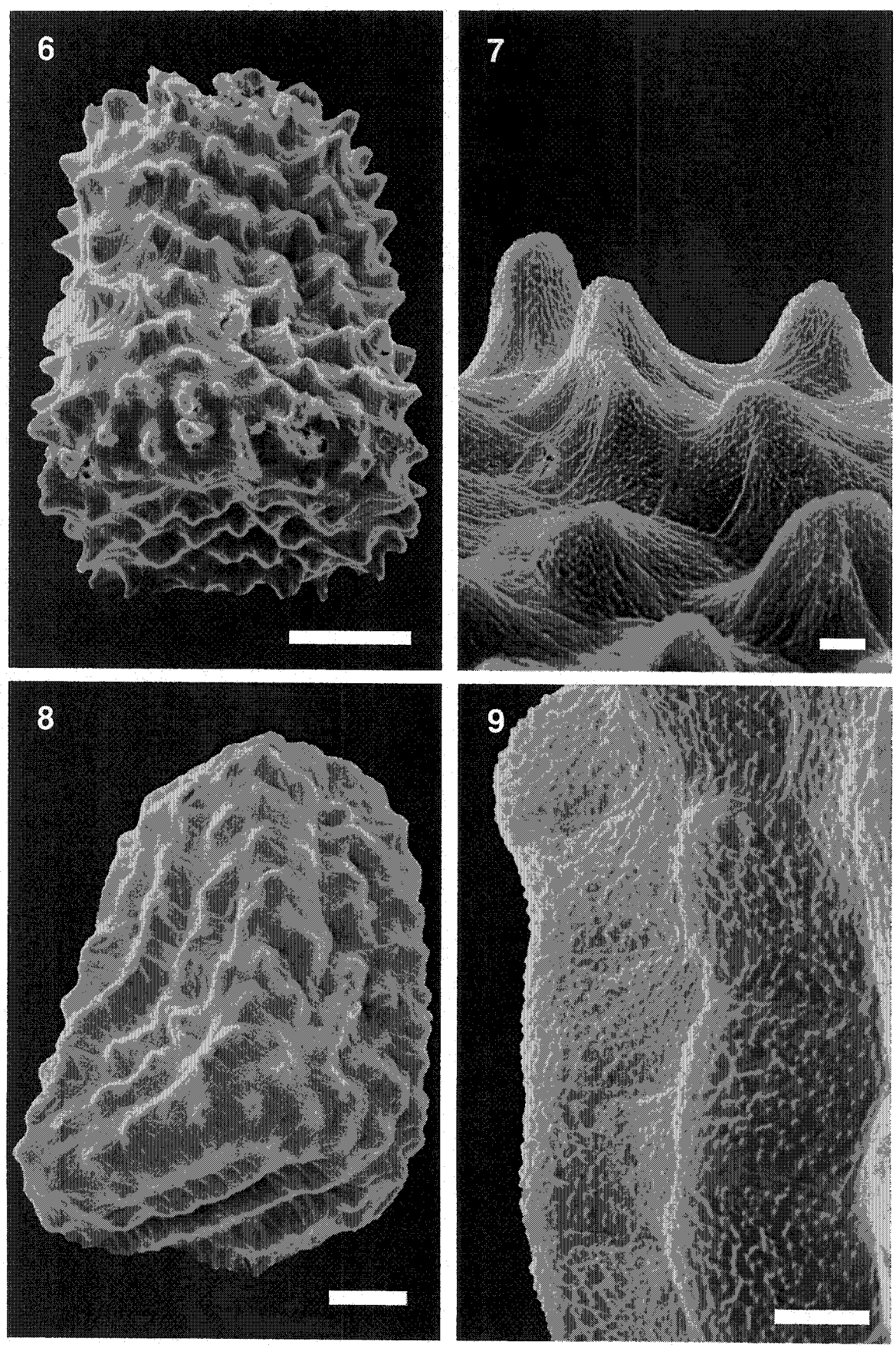
Figures 6 - 9: Scanning electron micrographs of seeds of *Nuttallanthus texanus*.

Figure 6: Oblique lateral view of seed, hilum to top (Crawford 27). Scale = 100 μ m.

Figure 7: Expanded view of seed (Crawford 27). Scale = 10 μ m.

Figure 8: Oblique lateral view of seed, hilum to top (Crawford 23). Scale = 100 μ m.

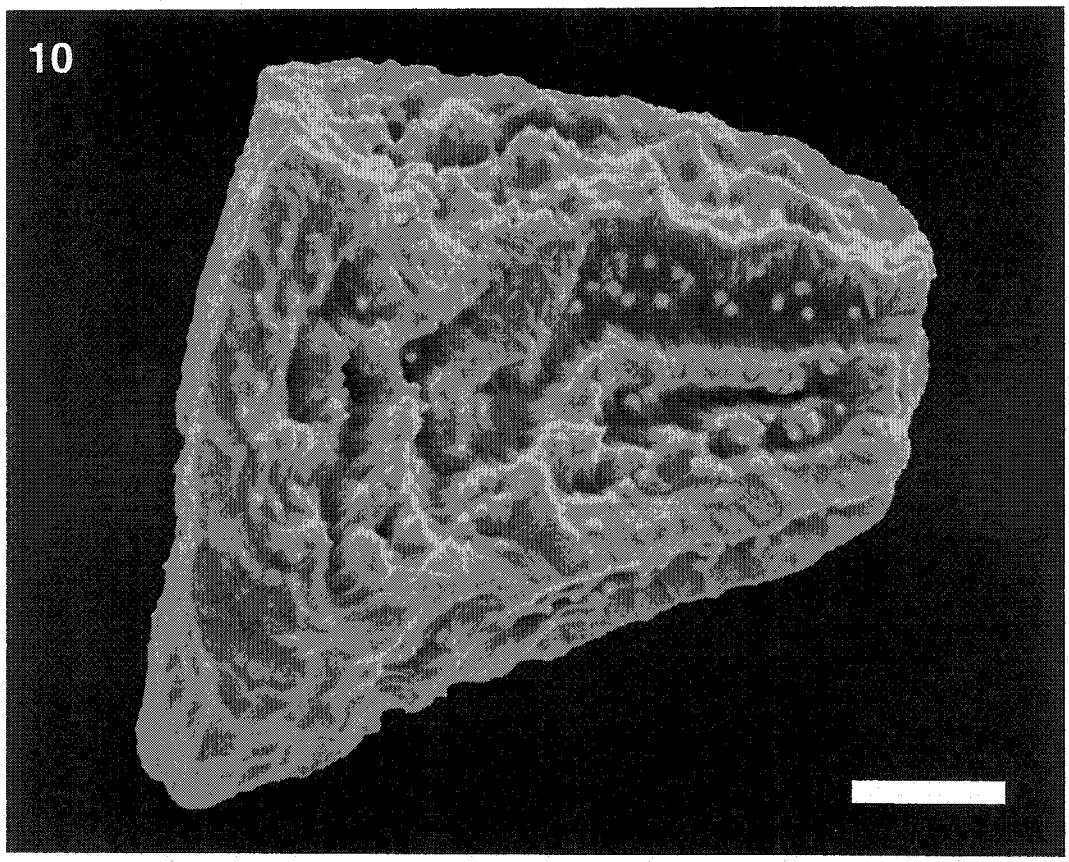
Figure 9: Expanded view of seed surface (Crawford 23). Scale = 10 μ m.


6
7
8
9

Figures 10 and 11: Scanning electron micrographs of seeds of *Nuttallanthus floridanus*.

Figure 10: Lateral view of seed, hilum to top (Godfrey 80417). Scale = 100 μ m.

Figure 11: Oblique view of distal end of seed (Godfrey 80417). Scale = 10 μ m.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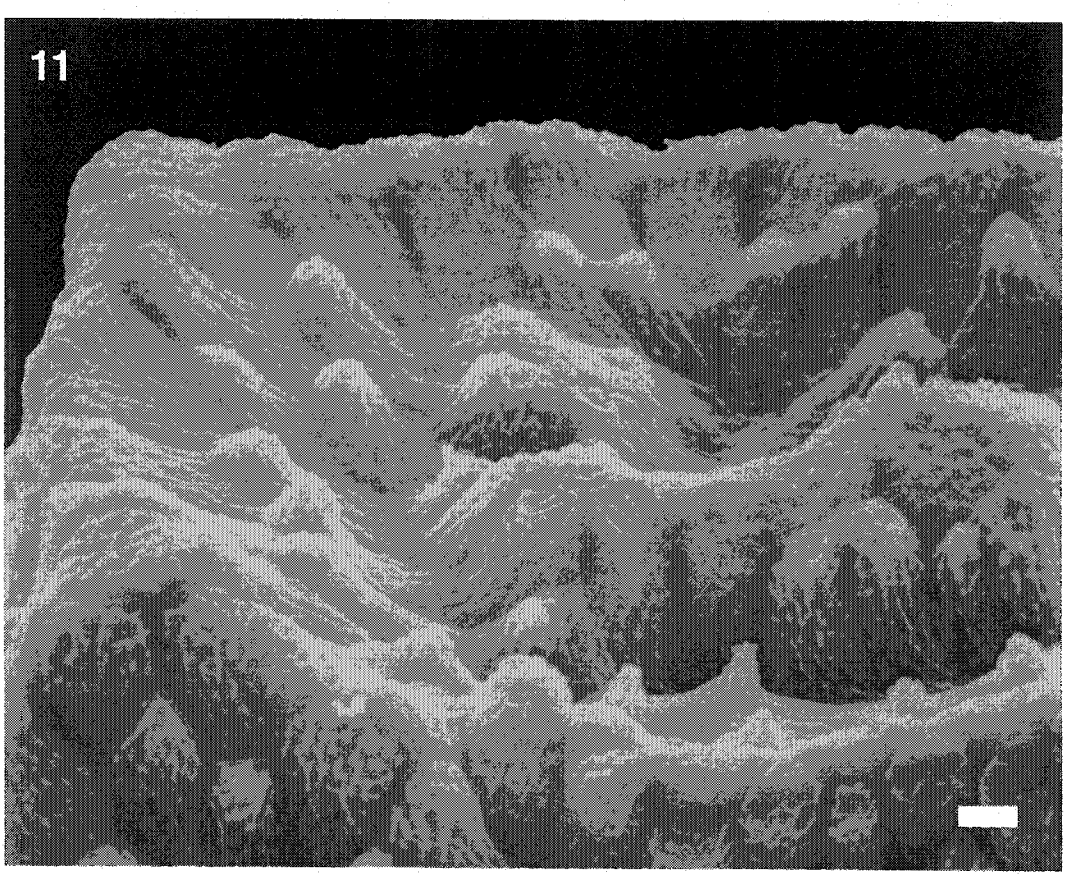
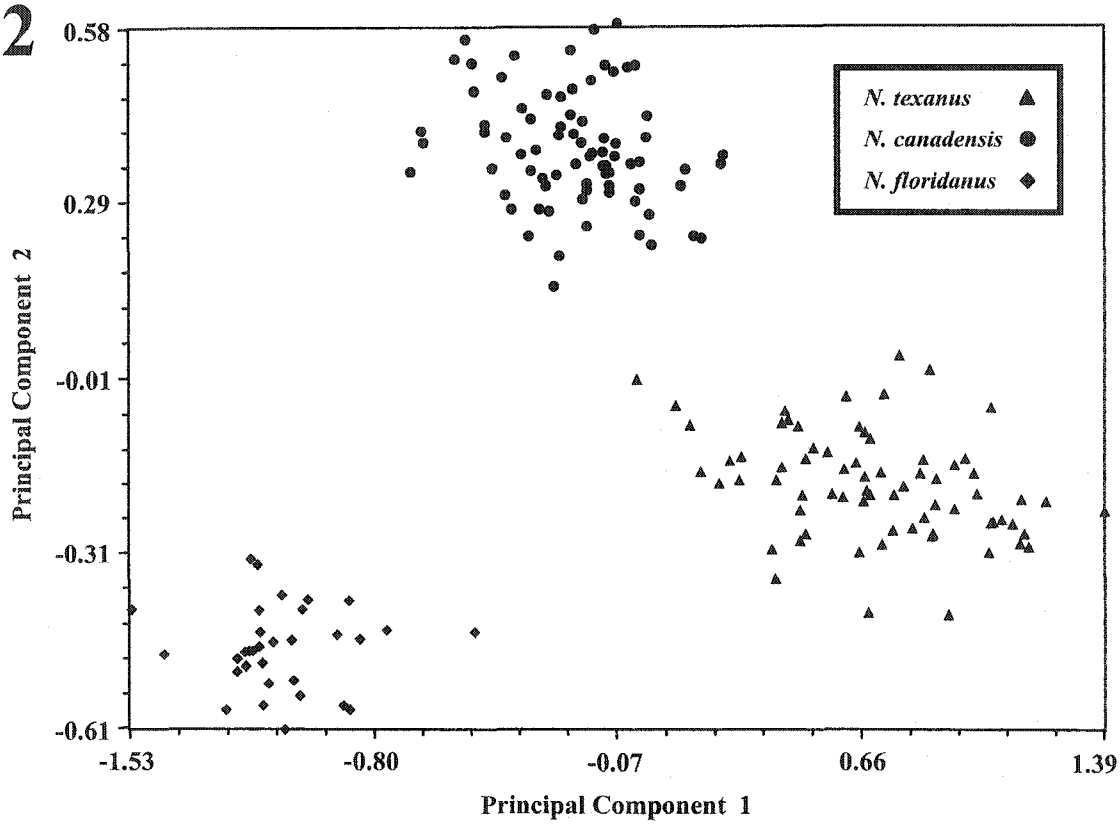

11

Figure 12 and 13. Two-dimensional plots of principal component analysis (PCA) of characters taken from individuals of three *Nuttallanthus* species.

Figure 12. PCA of 35 reproductive and vegetative characters taken from 179 individuals of three *Nuttallanthus* species. The first and second principal components account for 60.2% of the total variation in the data set. See Table 4 for loadings and eigenvalues.

Figure 13. PCA of 26 reproductive characters taken from 179 individuals of three *Nuttallanthus* species. The first and second principal components account for 73.3% of the total variation in the data set.

12


0.58
0.29
-0.01
-0.31
-0.61
Principal Component 2
-1.53
-0.80
-0.07
0.66
1.39
Principal Component 1
N. texanus
N. canadensis
N. floridanus

13

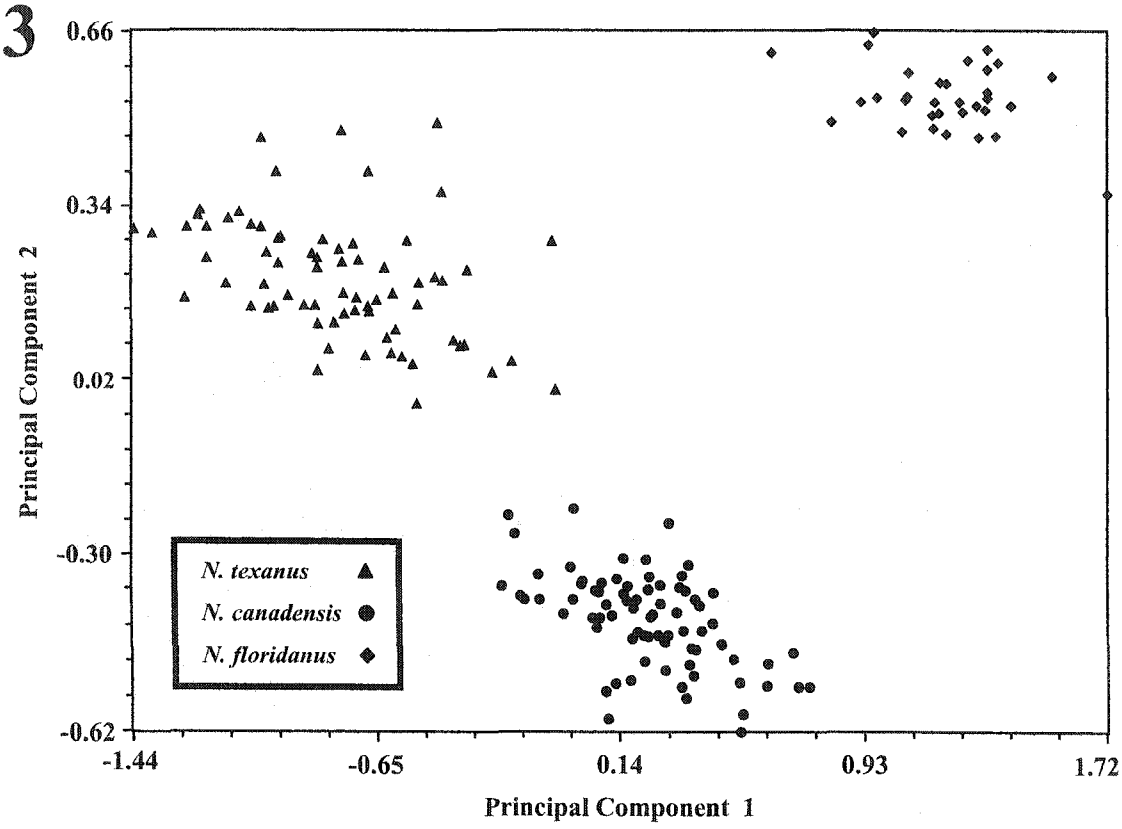
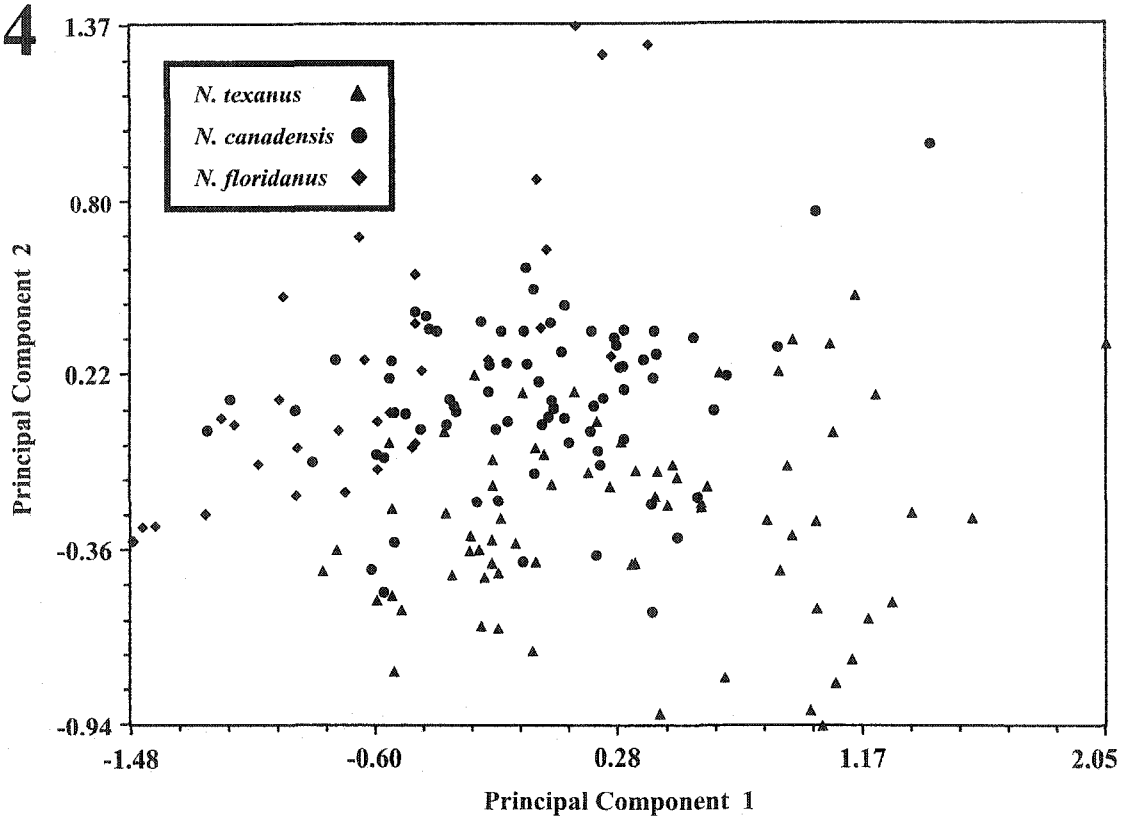

0.66
0.34
0.02
-0.30
-0.62
Principal Component 2
-1.44
-0.65
0.14
0.93
1.72
Principal Component 1
N. texanus
N. canadensis
N. floridanus

Figure 14 and 15. Two-dimensional plots of principal component analysis (PCA) of characters taken from individuals of three *Nuttallanthus* species.

Figure 14. PCA of 9 vegetative characters taken from 179 individuals of three *Nuttallanthus* species. The first and second principal components account for 56.1% of the total variation in the data set.

Figure 15. PCA of population means of 35 reproductive and vegetative characters taken from 50 populations of three *Nuttallanthus* species. The first and second principal components account for 70.6% of the total variation in the data set. Population numbers are listed in Table 1; population localities are depicted in Figure 1.

14


N. texanus
N. canadensis
N. floridanus
1.37
0.80
0.22
-0.36
-0.94
-1.48
-0.60
0.28
1.17
2.05
Principal Component 2
Principal Component 1

15

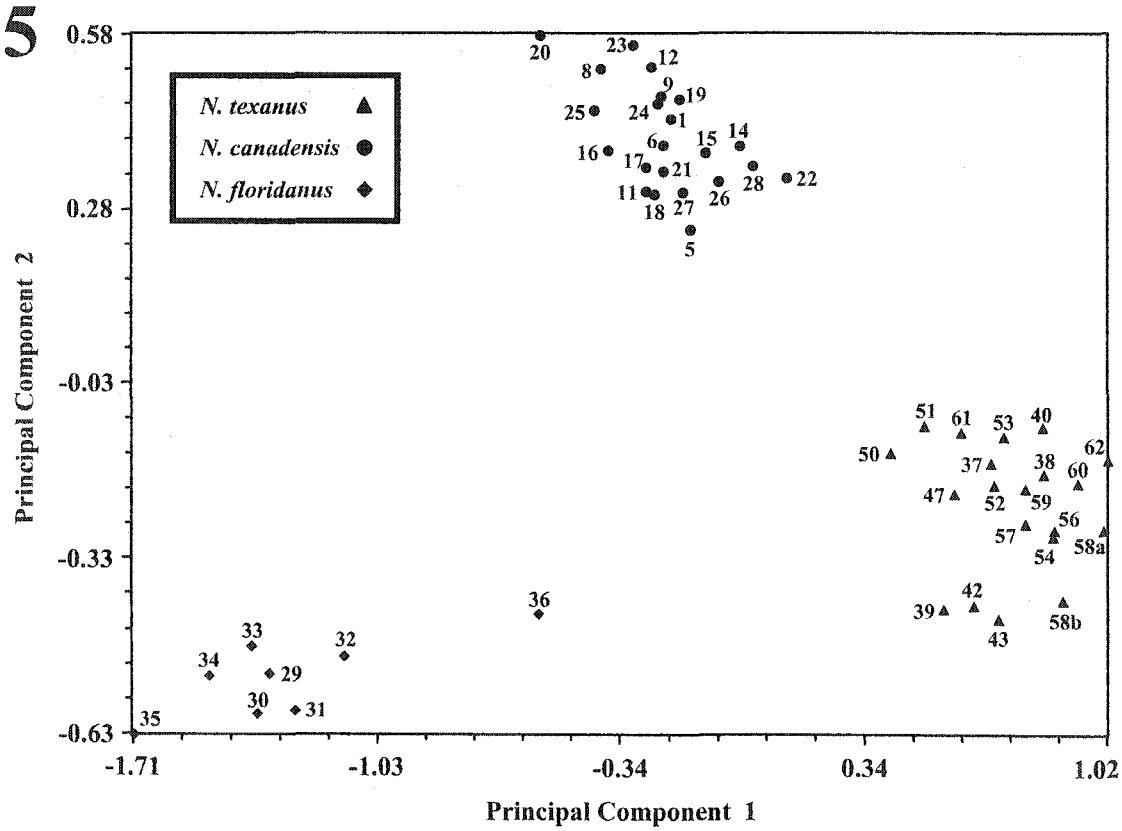

N. texanus
N. canadensis
N. floridanus
0.58
0.28
-0.03
-0.33
-0.63
-1.71
-1.03
-0.34
0.34
1.02
Principal Component 2
Principal Component 1

Figure 16. Three-dimensional plot of principal component analysis (PCA) using population means of 35 reproductive and vegetative characters taken from 50 populations of three *Nuttallanthus* species. The first three principal components account for 78.3% of the total variation in the data set. Population numbers are listed in Table 1; population localities are depicted in Figure 1.

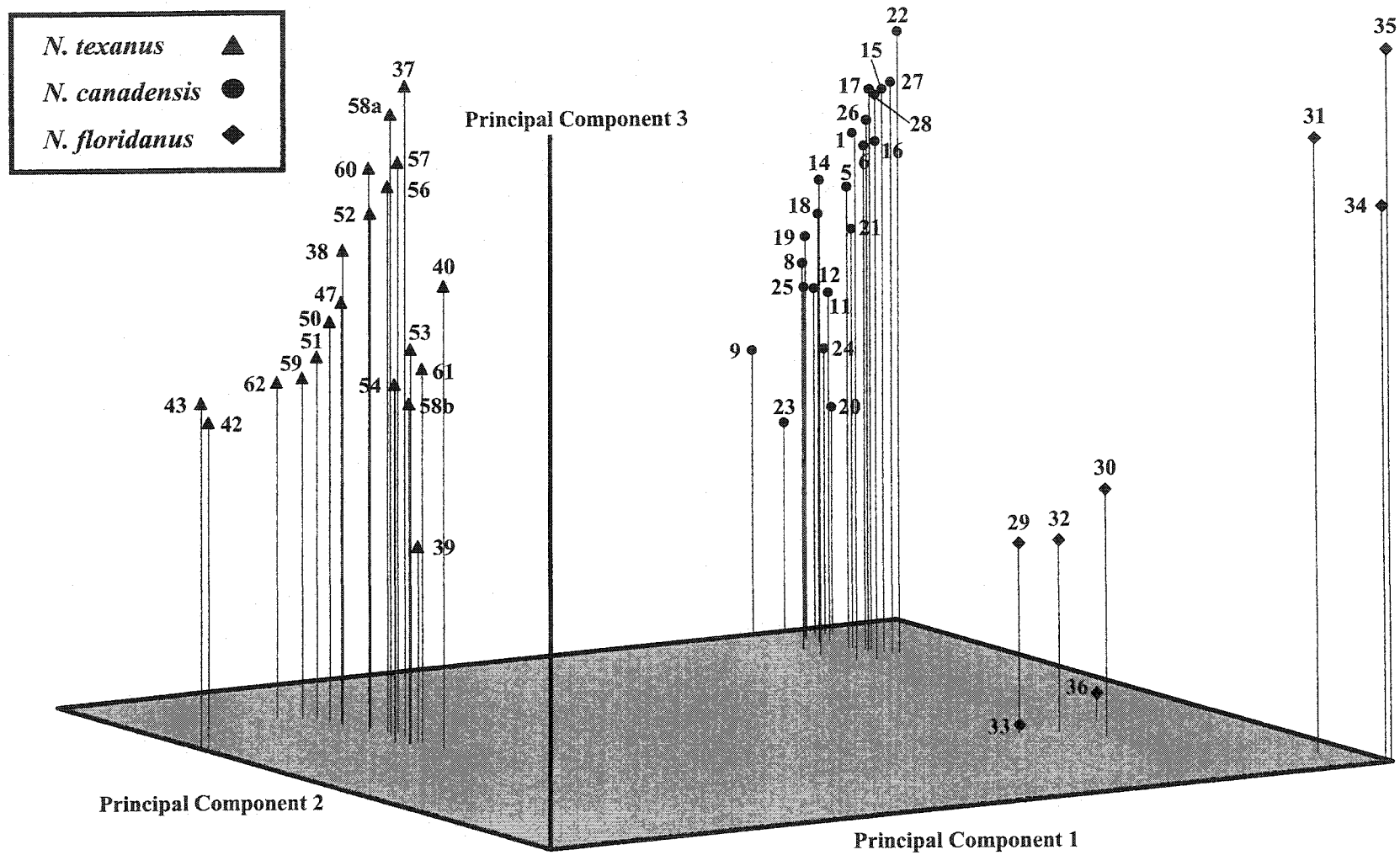
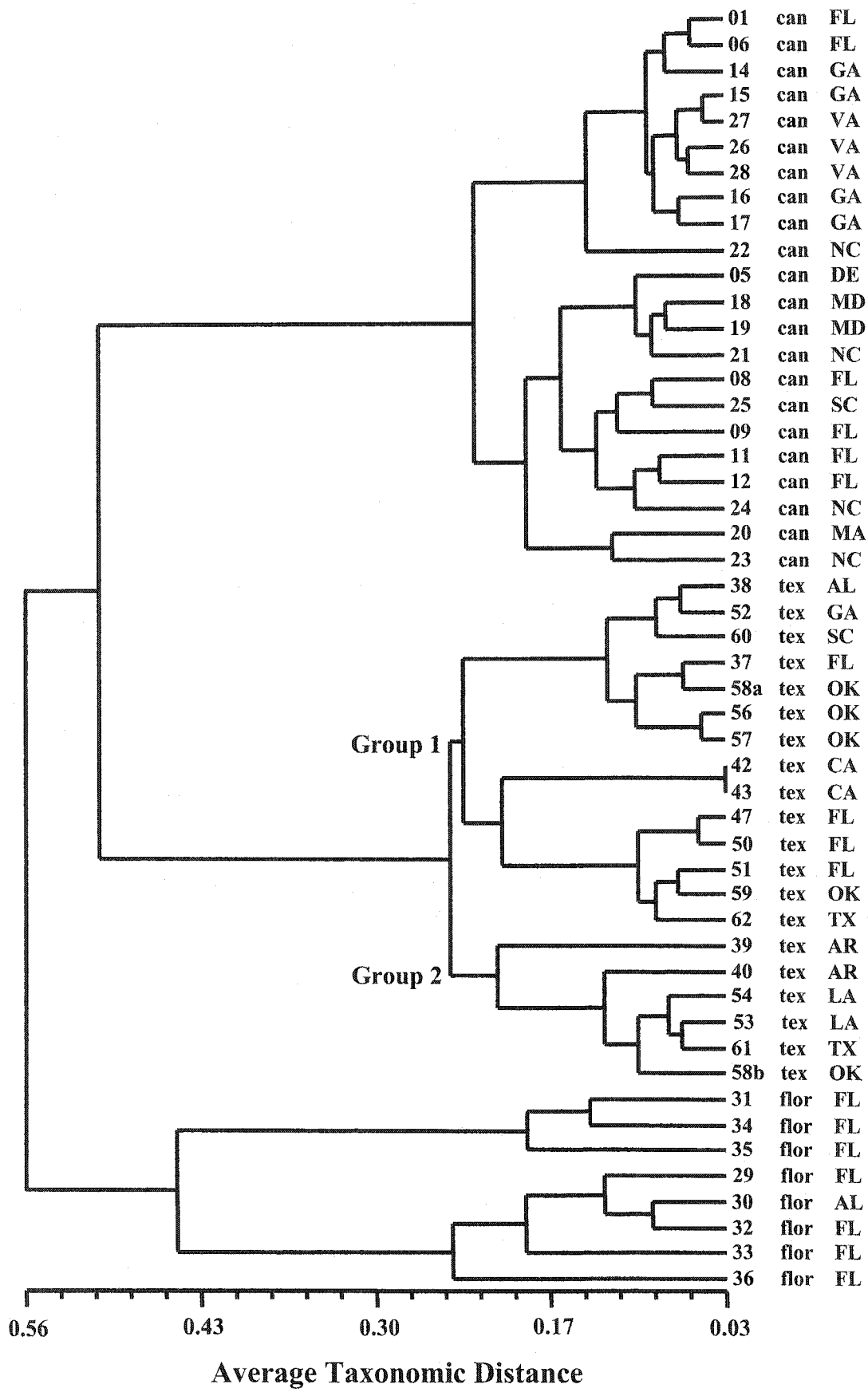

N. texanus
N. canadensis
N. floridanus
Principal Component 3
Principal Component 2
Principal Component 1
37
58a
60
57
56
52
38
40
47
50
51
53
59
62
61
54
43
58b
42
39
22
15
27
17
28
26
1
16
6
14
5
18
21
19
8
12
25
11
9
24
23
20
35
31
34
30
29
32
36
33

Figure 17. UPGMA phenogram derived from a matrix of average taxonomic distance coefficients among 50 populations of three *Nuttallanthus* species. Population numbers are listed in Table 1; population localities are depicted in Figure 1. The abbreviations *tex, can and flor* refer to populations of *N. texanus, N. canadensis* and *N. floridanus*, respectively, and are followed by the standard abbreviation for the state of collection. The cophenetic correlation is 0.925.


01 can FL
06 can FL
14 can GA
15 can GA
27 can VA
26 can VA
28 can VA
16 can GA
17 can GA
22 can NC
05 can DE
18 can MD
19 can MD
21 can NC
08 can FL
25 can SC
09 can FL
11 can FL
12 can FL
24 can NC
20 can MA
23 can NC
38 tex AL
52 tex GA
60 tex SC
37 tex FL
58a tex OK
56 tex OK
57 tex OK
Group 1
42 tex CA
43 tex CA
47 tex FL
50 tex FL
51 tex FL
59 tex OK
62 tex TX
39 tex AR
Group 2
40 tex AR
54 tex LA
53 tex LA
61 tex TX
58b tex OK
31 flor FL
34 flor FL
35 flor FL
29 flor FL
30 flor AL
32 flor FL
33 flor FL
36 flor FL
0.56
0.43
0.30
0.17
0.03
Average Taxonomic Distance

Appendix 1. Eigenvalues and loadings on the first three component axes from principal component analysis of morphological variation in three species of *Nuttallanthus*. Descriptions of characters are provided in Table 2.

Principal Component Axis	1	2	3
Eigenvalues	16.8	4.26	3.11
Variance (as percent of total)	48.0	60.1	69.1
		Loadings	
1. Plant height	0.563	-0.289	0.486
2. Stem diameter	0.531	-0.199	0.618
3. Number of fertile stems	-0.268	-0.113	0.605
4. Number of sterile stems	-0.152	0.158	0.329
5. Nodal density	0.033	0.484	-0.355
6. Sterile stem leaf length	0.454	-0.045	0.449
7. Sterile stem leaf width	0.445	0.126	0.484
9. Fertile stem leaf length	0.465	-0.090	0.621
10. Fertile stem leaf width	0.750	0.054	0.372
12. Inflorescence axis	-0.730	0.627	0.115
13. Floral bract length	0.750	0.067	0.208
14. Floral bract width	0.842	0.114	0.134
16. Flowering pedicel length	-0.060	0.837	0.074
17. Fruiting pedicel length	-0.380	0.781	0.236
18. Pedicel vestiture density	-0.886	-0.069	0.225
19. Adaxial calyx lobe length	0.770	0.133	0.304
20. Adaxial calyx lobe width at base	0.750	0.218	0.108
22. Abaxial calyx lobe length	0.859	0.084	0.226
23. Abaxial calyx lobe width	0.896	0.156	0.004
25. Corolla color	0.578	0.721	-0.033
26. Corolla length	0.898	0.141	-0.231
27. Adaxial corolla lobe length	0.904	0.122	-0.208
28. Adaxial corolla lobe width	0.738	-0.154	-0.162
30. Adaxial corolla sinus depth	0.767	-0.197	-0.208
32. Abaxial corolla lobe sinus depth	0.803	0.126	-0.300
33. Abaxial central corolla lobe width	0.844	-0.002	-0.263
35. Corolla tube length	0.841	-0.062	-0.125
36. Corolla tube diameter	0.849	0.130	0.002
39. Corolla spur length	0.881	-0.152	-0.260
40. Corolla spur diameter	0.805	-0.125	-0.169
43. Capsule length	0.803	-0.022	0.116
44. Capsule diameter	0.826	-0.132	-0.024
46. Acute, entire longitudinal ridges on seed	0.821	0.443	-0.079
47. Low, obtuse, sinuate ridges on seed	0.439	-0.632	-0.317
48. Acute, discrete multicellular tubercles on seed	-0.071	-0.840	0.179