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## Documentation of the Chromosome Number for *Zigadenus glaberrimus* (Liliales: Melanthiaceae) and its Significance in the Taxonomy of Tribe Melanthieae

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**Abstract**—The monotypic genus *Zigadenus* occupies a critical position in the evolution of Melanthieae as sister to the clade comprising the rest of the genera in the tribe. Meiotic ( $n = 27$ ) and mitotic ( $2n = 54$ ) chromosome counts for *Zigadenus glaberrimus* documented here do not support a long-standing tentative report of  $2n = 52$ . The likely base chromosome number of the tribe and significance of chromosome numbers as generic synapomorphies are discussed in reference to these newly recorded counts for *Zigadenus glaberrimus*, a likely hexaploid.

**Keywords**—Chromosomal evolution, Cytology, Phylogeny, Polyploidy.

Tribe Melanthieae (Liliales: Melanthiaceae) comprises seven genera (ca. 68–98 species) of predominately woodland and/or alpine perennial herbs occurring mainly in the temperate to Arctic zones of the Northern Hemisphere: *Amianthium* A. Gray (one species), *Anticlea* Kunth (ca. 11 species), *Schoenocaulon* A. Gray (25–27 species), *Stenanthium* (A. Gray) Kunth (five species), *Toxicoscordion* Rydb. (ca. eight species), *Veratrum* L. s.l. (17–45 species), and *Zigadenus* Michx. s.s. (one species). These generic circumscriptions are supported by analyses of *trnL-F* (plastid) and ITS (nuclear ribosomal) DNA sequence data (Zomlefer et al. 2001, 2003, 2006a, b). A significant consequence of these molecular studies was the reassessment of the traditional *Zigadenus* s.l., a poorly defined assemblage with a complex taxonomic history involving several proposed segregate genera (summaries in Zomlefer 1997 and Zomlefer et al. 2006a). These taxa share a somewhat similar scapose habit, usually with a bulb and racemes of small white flowers typically measuring five to 20 mm in diameter. Contemporary treatments (e.g. Schwartz 2002) have generally accepted the monotypic segregate *Amianthium* with the remaining ca. 25 species maintained in *Zigadenus* s.l. Based on these molecular data, however, *Zigadenus* s.l. is polyphyletic and forms five strongly supported clades (Fig. 1), each correlating with certain geographical distribution, morphological characters, and chromosome number (Zomlefer et al. 2001).

The reinstatement of segregate genera *Amianthium*, *Anticlea* and *Toxicoscordion* and the redefinition of *Stenanthium* result in a monotypic *Zigadenus*, a circumscription advocated historically by several botanists (e.g. Rydberg 1903; Small 1903, 1933; Gates 1918). The type (and only) species, *Z. glaberrimus* Michx. (sandbog death camas), is restricted to savannas, flatwoods, and bogs along the coastal plain of the southeastern U. S. A., from southeastern Virginia through the Carolinas, southern Georgia, and panhandle Florida, west to coastal Alabama (Fig. 2A). In phenetic analyses of morphological characters (Ambrose 1975, 1980; Schwartz 1994), *Z. glaberrimus* is the most isolated species of those studied in the tribe. Walsh (1940) suggested that *Z. glaberrimus* represented the ancestral form in the complex, based on the horizontal “woody” rootstock, suffrutescent stem, and relatively large flowers (to 30 mm diam; Fig. 2B) in a loose panicle inflores-

cence. Autapomorphies for this distinctive species (discussed in Zomlefer 1997; Zomlefer et al. 2006a) include a rhizome lacking a bulb, two ovate nectar glands per tepal (Fig. 2B), and several anatomical features (foliar stomata with two aperture lips, distinct root exodermis, bracteolate pedicels, and dense tannin-like inclusions; Ambrose 1975). In addition, a tentative chromosome count of  $2n = 52$ , which is unique for Melanthiaceae, has been reported by Preece (1956) but not verified.

Chromosome number is a significant and likely an invariable apomorphy for genera of Melanthiaceae (Zomlefer et al. 2001, 2006a), and members of tribe Melanthieae have a range of chromosome counts (Fig. 1), indicating several possible chromosomal restructuring events in the evolution of the tribe. *Zigadenus* in particular occupies a divergent position in the phylogeny of Melanthieae as sister to the rest of the tribe. The purpose of our study is to document this potential autapomorphy for *Zigadenus* s.s., particularly in reference to a current investigation of chromosomal evolution in the family Melanthiaceae.

### MATERIALS AND METHODS

**Meiotic Chromosome Number**—Pollen mother cells (PMCs; microcytes) were prepared according to the general protocols outlined by Jones and Luchsinger (1986). Young flower buds of *Zigadenus glaberrimus*, collected in Apalachicola National Forest (Liberty County, Florida) by the first author at 10:00 AM, were fixed immediately in the field with modified Carnoy's solution comprising 4 parts chloroform: 3 parts absolute (ethyl) alcohol: 1 part glacial (anhydrous) acetic acid. Immature flower buds (ca. 5.0 mm long; Fig. 3C) enclosed anthers (ca. 1.8 mm long; Fig. 3D) with PMCs undergoing optimal stages of meiosis for chromosome counts (late prophase I [diakinesis] to metaphase I/early anaphase I). Anthers were dissected from the buds, placed on a slide with several drops of diluted (ca. 1%) acetocarmine stain, and gently macerated. After application of a cover slip, the slide was placed between blotters and subjected to pressure. Slides were mounted in euparal for future reference. Well-spread metaphase I chromosomes were traced by the first author under a Leica DMLB Research Microscope with a camera lucida attachment. The herbarium voucher specimen, Zomlefer 803, is deposited at GA, and a duplicate, at FLAS (Appendix 1).

**Mitotic Chromosome Number**—Several live plants of *Zigadenus glaberrimus* were collected by Andrew Scott Walker in Hoke County, North Carolina, and transplanted to pots maintained at the Department of Plant Biology Greenhouse Facility at the University of Georgia. Root tips were prepared for chromosome spreads according to the protocols

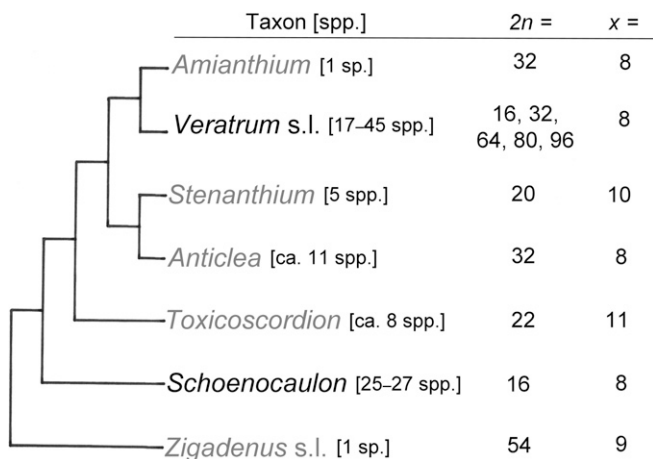


FIG. 1. Phylogenetic relationships and reported chromosome numbers for the genera of the tribe Melanthieae. Cladogram modified from Zomlefer et al. (2001) and Zomlefer and Smith (2002), based on ITS and *trnL-F* sequence data; chromosome numbers from references summarized in Zomlefer (1997) and the new report for *Zigadenus glaberrimus*, verified here. Gray type = segregate genera of the polyphyletic *Zigadenus* s. l. complex (Zomlefer and Judd 2002).

of Kato et al. (2004). Actively growing root tips (1 cm long) were harvested at 9:00 AM, placed in a nitrous oxide chamber at 10 atm for 3 hrs, fixed in ice cold 90% acetic acid for 10 min, and washed twice with ice cold 1 × citric buffer (5 mM sodium acetate, 5 mM EDTA, adjusted to pH 5.5) for 10 min. The tip of the root cap was removed, and ca. 1–1.5 mm of the remaining distal end (opaque region comprising actively dividing cells) was transferred to a tube containing 20  $\mu$ L of 0.1 g pectolyase Y-23, 0.2 g cellulase Onozuka R-10, and 9.7 g 1 × citric buffer (10 mL total stock

solution) and incubated for 30 min at 37°C. Incubated samples were subsequently plunged into ice to stop digestion. Root tips were then rinsed with ice cold TE (Tris-EDTA) buffer solution [10 mM Tris, 1 mM EDTA adjusted to pH 8.0 with HCl], followed by three washes with 100% ethanol and gentle maceration in 30  $\mu$ L of 3 parts acetic acid: 1 part ethanol. Suspended cells (6–8  $\mu$ L) were placed on slides, dried in a humid chamber, and examined with a compound microscope to identify the best chromosome spreads. Slides were fixed with VECTASHIELD® mounting medium with DAPI (4',6-diamidino-2-phenylindole dihydrochloride) fluorescent stain (Vector Laboratories, Burlingame, California) prior to application of a cover slip. Well spread metaphase chromosomes were photographed under a Zeiss Axio Imager M1 microscope using SlideBook™ 5.0 imaging software (Denver, Colorado). The herbarium voucher specimen, Walker s.n. [19 Aug. 2011], is deposited at GA (Appendix 1).

## RESULTS AND DISCUSSION

The meiotic and mitotic chromosome numbers of  $n = 27$  (Fig. 3 E) and  $2n = 54$  (Fig. 3F, G) for *Zigadenus glaberrimus* are documented with plants from populations in Liberty County, Florida, and Hoke County, North Carolina, respectively. This study challenges the earlier mitotic report of “52?” by Preece (1956, p. 17), who also noted the unusual chromosome number in comparison to other species in the tribe. The difference in counts may be due to difficulties with adequately spreading the mitotic chromosome preparation in this particular species. In our case, the haploid count corroborates the diploid number from a distant population.

Chromosome number is a useful taxonomic character for the superficially similar genera within tribe Melanthieae (Fig. 1), especially the synapomorphic  $2n$  numbers 20 for *Stenanthium* (Zomlefer and Smith 2002) and 22 for *Toxicoscordion* (Zomlefer 2003; McNeal and Zomlefer 2010). A probable base chromosome number of  $x = 8$  has often been postulated for tribe Melanthieae (summary in Zomlefer et al. 2006a), and multiples of this number are prevalent in other genera of the tribe: (Fig. 1; Sen 1975; Lowry et al. 1987; Tamura 1995; Zomlefer 1997): *Amianthium* ( $2n = 32$ ), *Anticlea* ( $2n = 32$ ), *Schoenocaulon* ( $2n = 16$ ), and *Veratrum* (including *Melanthium*;  $2n = 16, 32, 64, 80, 96$ ). *Zigadenus*, another exception to an octomeric count, is of particular interest due to its significant position in the evolution of Melanthieae as the functional outgroup of the tribe, and the verified count allows formal reassessment of the base number for tribe Melanthieae.

Our chromosome number has been included in a recent reconstruction of ancestral chromosome numbers for major clades within Melanthiaceae (Pellicer et al. 2014). Bayesian and maximum likelihood analyses with ChromEvol v. 1.3 (Mayrose et al. 2010) support nine as the likely base (haploid) chromosome number for tribe Melanthieae, and eight for the sister-clade to *Zigadenus glaberrimus*, which includes the remaining genera in the tribe. These hypothesized basic numbers indicate the prevalence of polyploidy (*Amianthium*, *Anticlea*, *Veratrum*, *Zigadenus*) and/or aneuploid variation (*Stenanthium*, *Toxicoscordion*) within the tribe.

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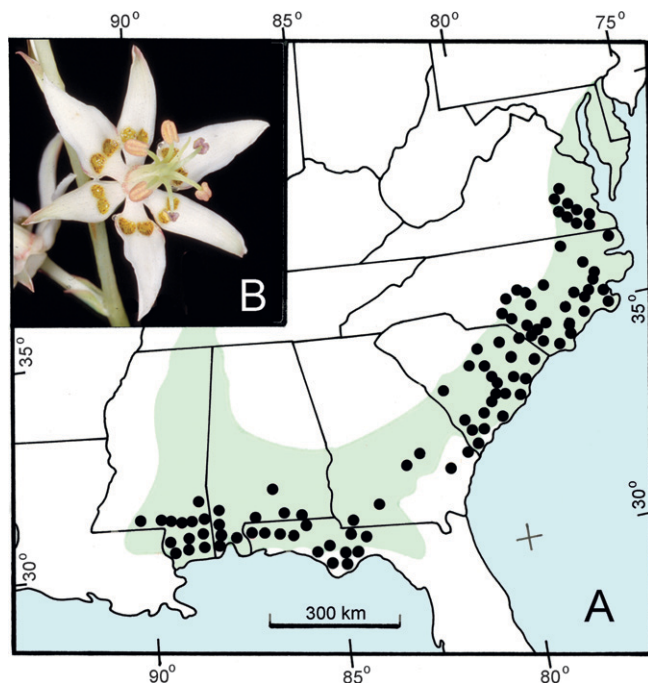


FIG. 2. Distinctive characteristics of *Zigadenus glaberrimus*. A. General distribution map. Dots = herbarium specimen locality data from AUA, BRIT/SMU, FLAS, GA, LSU, MISS, MISSA, USMS, VDB, and VSC, and voucher citations in Preece (1956), Johnson (1969), Liu et al. (2006), Kral et al. (2013), Urbatsch et al. (2013), Virginia Botanical Associates (2013), and Wunderlin and Hansen (2013); light green shading = Middle Atlantic Coastal Plain and Southeastern Plains Level III Ecoregions (US EPA 2013). B. Flower (note paired perigonal nectaries)  $\times 1.4$ . Photo credit: W. Mark Whitten (Zomlefer 696a, FLAS).

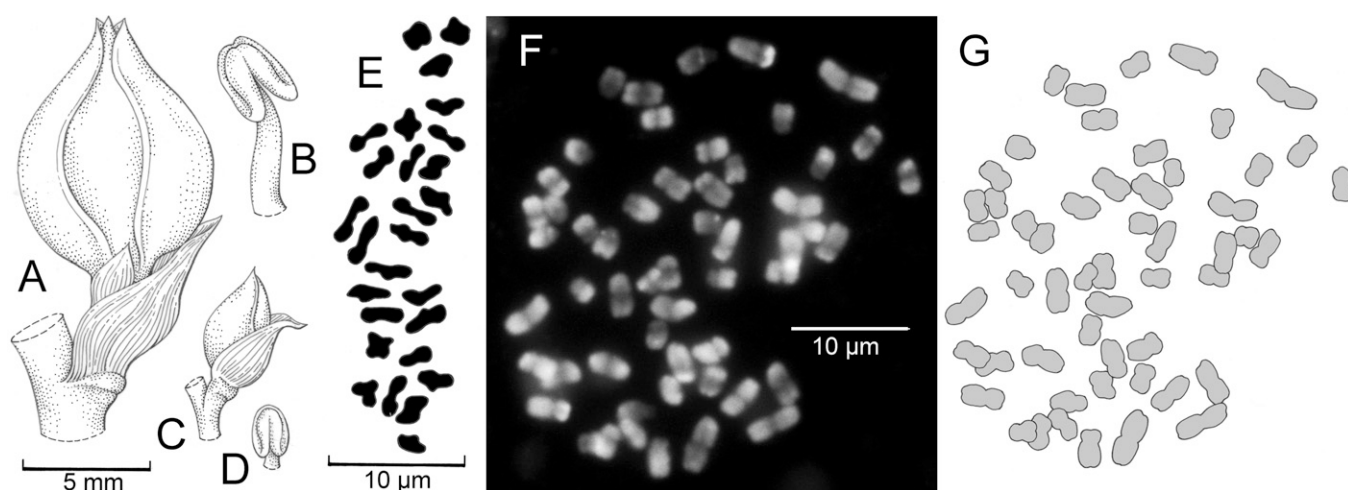


FIG. 3. Meiotic and somatic chromosome counts for *Zigadenus glaberrimus*. A–E. Meiotic chromosome count ( $n = 27$ ). A. Mature flower bud, just prior to anthesis. B. Anther from bud depicted in A. C. Flower bud stage containing immature anthers suitable for meiotic chromosome counts (compare to A). D. Optimal anther size (compare to B) with pollen mother cells undergoing late prophase I [diakinesis] to metaphase I/late anaphase I stages of meiosis. E. Meiotic chromosomes (from pollen mother cells), metaphase I. F–G. Mitotic chromosome count ( $2n = 54$ ). F. Photograph of metaphase chromosomes in root tip cell. G. Outline of F. A–E from Zomlefer 803 (GA); F–G from Walker s. n. 19 Aug 2011 (GA).

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- APPENDIX 1. Voucher information for the chromosome numbers of *Zigadenus glaberrimus* reported in this study.
- Meiotic count.** U. S. A. North Carolina: Hoke Co., Fort Bragg, 35.07876° N, 79.22001° W, 19 Aug 2011, *Walker s. n.* (GA).
- Mitotic count.** U. S. A. Florida: Liberty Co., Sumatra, Apalachicola National Forest, Forest State Road 181, 3.2 mi from intersection with FL 379, 30.05519° N, 84.94983° W, 19 Jun 2002, *Zomlefer 803* (GA).