The utility of DNA markers for delimiting and identifying species of *Gamochaeta* (Compositae) in the eastern United States

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ABSTRACT

Gamochaeta (Compositae) is a genus of 50–80 herbaceous species with a center of diversity in South America and with several common, generally weedy species in the eastern United States. Historically, some authors recognized as few as one species in the eastern United States while others have recognized as many as eight. These increased numbers are a product of both naturalization of nonnative species and recognition of new taxa. A study was undertaken with DNA markers to assess whether the putative species of this region exhibit genetic differences and to determine if DNA markers may be useful tools for identification. One nuclear and one plastid region of DNA were sequenced, and unique nuclear markers were found for all eight recognized species and unique plastid markers were found for five of the eight species. These results support the species hypotheses for entities in this region. Unlike other genera of the Compositae (e.g., *Solidago*) that possess little genetic differentiation in commonly used barcoding regions of DNA, the species of *Gamochaeta* in the eastern United States can be identified using a single nuclear marker, and several may be identified with a single plastid marker. Given that species of *Gamochaeta* are naturalizing in many places on Earth, these data will provide an important resource for identification.

Key words: Asteraceae, cudweeds, DNA barcoding, Gnaphalieae, *Gnaphalium*, species delimitation

INTRODUCTION

Gamochaeta Wedd. (Compositae: Gnaphalieae) consists of about 50–80 species of herbaceous flowering plants with a center of diversity in South America (Anderberg 1991; Bayer et al. 2007; Cabrera 1961; Deble and Cardoso Marchiori 2007; Freire and Iharlegui 1997; Nesom 2006; Urtubey et al. 2016). *Gamochaeta* is related to *Gnaphalium* L. (Anderberg 1991; Funk et al. 2009; Urtubey et al. 2016), a genus in which it was formerly placed, but is distinguished from it and other genera of tribe Gnaphalieae by its combination of relatively small heads (2.5–5.0 mm) in spike-like clusters, concave post-fruiting receptacles, cypselae with myxogenic (mucilage-producing) hairs, and pappus bristles basally connate into a ring and released as a single unit (Anderberg 1991; Bayer et al. 2007; Cabrera 1961; Nesom 2006), the latter feature the source of its scientific name.

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Morphological characteristics such as leaf shape, distribution of indument, and shape and size of the phyllaries are used to delimit, recognize, and identify the putative species of this genus (Cabrera 1961; Freire et al. 2016; Nesom 2006).

Commonly known as "cudweeds," "everlastings," or "rabbit-tobaccos," species of *Gamochaeta* are often found in great abundance in the eastern part of the United States, especially in lawns and ruderal habitats. The species appear to be expanding in range in both the United States (e.g., Alford 2012; Atha et al. 2016; Nesom 1999, 2004a, 2007; Simmons et al. 2008; Vincent et al. 2011) and other places of the world (e.g., Galasso et al. 2018; Hamel and Azzouz 2018; Iamonico 2012; Ji et al. 2014; Shahid 2014; Soldano 2000). In addition, new species continue to be described, primarily in the tropical Americas (e.g., Deble et al. 2006; Freire and Urtubey 2013) but also in the United States (Nesom 2004b, 2022).

In the eastern United States, cudweeds have historically been treated as a single, highly variable species (e.g., Gleason and Cronquist 1991) or as a small number of species or varieties (e.g., Ahles 1968; Godfrey 1958; Small 1933), but recent treatments (e.g., Nesom 1990, 2004a, 2004b, 2006, 2022; Weakley and Southeastern Flora Team 2022) recognize eight species (Table 1). An additional four species are recognized from Texas and the Pacific West (Nesom 2006). The number of species differs by treatment for two main reasons: (1) the naturalization of nonnative species and (2) the recognition of new species within the existing variation of broadly circumscribed, known species. To make matters complicated, species boundaries have been interpreted differently (e.g., Freire et al. 2021; Nesom 1990, 2004a, 2022), and several taxa have had misapplied names or complex nomenclatural issues (e.g., Nesom 2006, 2022; Pruski and Nesom 2004), resulting in (multiple) name changes. Or, as Nesom (1990: 189) succinctly put it, "[T]he application of names to North American 'gamochaetoid' species has been extremely uneven."

The confusion about names and species circumscriptions may also be attributed to a lack of material for study from other places in the world from whence new introductions come, the lack of quantitative sampling and molecular data for comparison among species, and a reticence to rely on the microscopic characters often used to distinguish species in some groups of Compositae. Here we address the second issue and present DNA data from the eight species recognized in the eastern United States, but our understanding of the genus could also benefit from further study of chromosome numbers, fruit morphology, and environmental effects on morphological variation. For example, preliminary results from scanning electron microscopy indicate that several of these species can be recognized by their cypsela size, ornamentation, and distribution of myxogenic hairs (Alford, unpubl. data), features used in related taxa (Abid and Qaiser 2008).

Based on observations of morphological discontinuity, multiple species of *Gamochaeta* have been hypothesized and are now widely recognized for the eastern United States (Table 1). However, whether some (or all) of the morphological diversity is infraspecific, is environmental, or represents features associated with different species is mostly untested. Here we test the hypothesis that distinctive DNA patterns will be associated with entities recognized by morphology. The null hypothesis is that DNA variation will be independent of morphology.

Table 1. Species of *Gamochaeta* from the eastern United States as recognized by various, commonly used references (floras). *=nonnative, °=possibly nonnative (based on Nesom 2004a). Prior to Nesom (1990), the species in the United States were usually treated as part of *Gnaphalium*. *Gamochaeta impatiens* represents what was called *C. coarctata* by Nesom (2006) and *G. americana* by Freire et al. (2021).

Species	Small 1933	Godfrey 1958	Ahles 1968	Gleason & Cronquist 1991	Nesom 2006; Weakley and Southeastern Flora Team 2022
°*Gamochaeta antillana* (Urb.) Anderb.	+	+	+ (as a variety)	–	+
°*Gamochaeta argyrinea* G.L. Nesom	–	–	–	–	+
°*Gamochaeta calviceps* (Fernald) Cabrera	–	+	–	–	+
**Gamochaeta chionesthes* G.L. Nesom	–	–	–	–	+
**Gamochaeta impatiens* G.L. Nesom	–	+	+ (as a variety)	–	+
°*Gamochaeta pensylvanica* (Willd.) Cabrera	+	+	+ (as a variety)	–	+
Gamochaeta purpurea (L.) Cabrera	+	+	+	+	+
**Gamochaeta simplicicaulis* (Willd. ex Spreng.) Cabrera	–	–	–	–	+

MATERIALS AND METHODS

Species of *Gamochaeta* and its close relative *Facelis* Cass. (Anderberg 1991) were collected from the eastern United States, Oregon, and Italy from 2007–2019 (Appendix). *Gamochaeta ustulata* (Nutt.) Holub from the west coast of the United States was included because Nesom (2004a, b) suggested that it may be the closest relative of the eastern *G. argyrinea* G.L. Nesom, a species he newly described from within the then-recognized variation of *G. purpurea* (L.) Cabrera. The collections were identified using the taxonomic key of Nesom (2006) to distinguish the finest divisions of morphological diversity (greatest number of putative species), although his *G. coarctata* (Willd.) Kerguélen is here recognized as *G. impatiens* G.L. Nesom (Nesom 2022). Freire et al. (2021) presented

evidence that *G. americana* (Mill.) Wedd. and *G. coarctata* are the same species, but Nesom (2022) argued that Freire et al.'s sampling was inadequate from the eastern United States and overlooked the species commonly known by the (misapplied) name of *G. coarctata*. Leaf fragments from these recently collected specimens were removed for DNA extraction, and the collections were deposited in the herbarium of the University of Southern Mississippi (USMS). Duplicates of the specimens from Georgia were deposited at VSC and those from South Carolina at US.

Total DNA extraction was performed using a DNeasy Plant Mini Kit (Qiagen, Valencia, CA). Approximately 15–20 mm^2 of clean leaf tissue was used. The instructions were modified in that 500 µL of Buffer AP1 was added to the plant tissue in a mortar, and the plant tissue was then pulverized using a pestle at room temperature until all that remained was mostly a uniform greenish liquid. The RNase A solution was not added.

After the DNA extraction was complete, the nuclear internal transcribed spacer (ITS) and the plastid *trnH-psbA* intergenic spacer were amplified in 50 µL reactions. Both regions are known for their high variability, even among closely related species (Bolson et al. 2015; Schilling and Floden 2012; Shaw et al. 2007). The amplification reaction consisted of 18 µL of water, 25 µL of TaKaRa ExTaq Premix (TaKaRa Bio USA, Madison, WI), 2.5 µL each of the forward and reverse primers, and 2.0 µL of the extracted DNA solution. Primers for ITS were ITS-4 and ITS-5 (White et al. 1990), and primers for *trnH-psbA* were $trnH^{GUG}$ (Tate and Simpson 2003) and psbA (Sang et al. 1997). The tubes were then placed in a Thermo PCR Sprint thermal cycler for polymerase chain reaction (PCR), with an initial three minutes at 94°C followed by 30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 60 s. After the 30 cycles, a final extension phase was set at 72°C for 5 minutes. More recent amplifications (those collected from Georgia, South Carolina, and Virginia) followed the protocol outlined in Samarakoon et al. (2013), which included the use of an enhancer of trehalose, bovine serum albumin, and polysorbate-20. Amplification products were visualized on 1.5% agarose gels for sufficient amplification and appropriate size. The DNA samples were then cleaned using a Qiaquick PCR Purification Kit (Qiagen). The DNA concentration of each sample was measured using a Nanodrop ND-1000 Spectrophotometer, utilizing the program NanoDrop 3.0.1 (Coleman Technologies, Orlando, FL).

The samples were sent to Eurofins MWG/Operon in Huntsville, Alabama, or Louisville, Kentucky, for sequencing. Chromatograms were checked for accuracy, and ambiguous base-pairs (bp) at the beginning or end of the sequences were deleted using Sequencher 4.7 (GeneCodes, Ann Arbor, MI). Forward and reverse sequences were joined together into contigs, and edited sequences were then exported as FASTA files. A few additional sequences whose source and identification could be confirmed with herbarium vouchers (Schilling and Floden 2012; Urtubey et al. 2016) were downloaded from GenBank (Sayers et al. 2022), and sequences were aligned in ClustalX 2.0.7 (Conway Institute, Dublin, Ireland) using default settings for comparison of homologous bps.

Aligned sequences were opened in WinClada 1.00.08 (K.C. Nixon, Cornell University), and a phylogenetic tree was inferred using *Facelis* as the outgroup. Phylogenetic analysis consisted of 10 sequential rachets (Nixon 1999), each with 20 iterations holding two trees per iteration, followed by a heuristic parsimony search of 500 replications, saving

10 trees per replication. Following the inference of phylogenetic trees, the unique sets of sequences and the related units on the trees were compared to the species of the eastern United States as recognized in Nesom (2006).

RESULTS

Sequences of DNA were newly obtained from 46 samples, representing all eight putative species of *Gamochaeta* in the eastern United States. Of the species sampled, all were represented by multiple collections except for *G. simplicicaulis* (Willd. ex Spreng.) Cabrera and the western *G. ustulata*. The nuclear ITS region did not amplify for *G. simplicicaulis*, but a sequence was available in GenBank and was included in the analysis. Five additional sequences of ITS were available in GenBank from Schilling and Floden (2012), who also used the circumscriptions of Nesom (2006).

The ITS matrix consisted of 42 individuals, and the *trnH-psbA* matrix consisted of 43 individuals. After trimming the ends of the sequences to minimize missing data, the aligned ITS sequences were 638 bp long, and the *trnH-psbA* sequences were 496 bp long. There was little DNA sequence variation within *Gamochaeta*, with only 15 informative substitution differences (2.3%) and one informative gap in ITS, and 21 informative substitutions (4.2%) and 8 gaps in the *trnH-psbA* spacer.

There were 11 unique sets of sequences in the ITS data, each corresponding to the outgroup or one of the recognized species of *Gamochaeta* in the eastern United States. Two unique sequences corresponded to *G. antillana* (Urb.) Anderb. Phylogenetic analysis of the ITS data resulted in 11 most parsimonious trees (MPTs) of length 17 and ensemble consistency index (CI; Kluge and Farris 1969) and ensemble retention index (RI; Farris 1989) of 1.00 (i.e., no homoplasy), which were simplified by collapsing all unsupported nodes (Figure 1).

There were nine unique sets of sequences in the *trnH-psbA* data, corresponding to the outgroup and five of the recognized species. Two unique sets of sequences were found for *Gamochaeta pensylvanica* (Willd.) Cabrera and *G. purpurea*. *Gamochaeta argyrinea*, *G. chionesthes* G.L. Nesom, *G. simplicicaulis*, and *G. ustulata* all had the same sequence. Phylogenetic analysis of the *trnH-psbA* data resulted in 81 MPTs of length 31 and CI and RI of 1.00, also simplified by collapsing all unsupported nodes (Figure 2). Character state changes were mapped onto the trees in Figures 1 and 2 with numbers representing the bp positions in the aligned matrices.

The tree inferred from analysis of the ITS data (Figure 1) indicated that each putative species of *Gamochaeta* in the eastern United States has a unique combination of DNA characters, with additional variation within *G. antillana*. The tree inferred from analysis of the *trnH-psbA* data (Figure 2) indicated that *G. antillana*, *G. calviceps*, *G. impatiens*, *G. pensylvanica*, and *G. purpurea* have unique combinations of DNA characters, with additional variation within *G. pensylvanica* and *G. purpurea*.

DISCUSSION

The hypothesis of multiple species of *Gamochaeta* in the eastern United States was affirmed. The unique nuclear DNA markers corresponded to each of the recognized species with additional variation within *G. antillana*. Phylogenetic analysis of the nuclear DNA

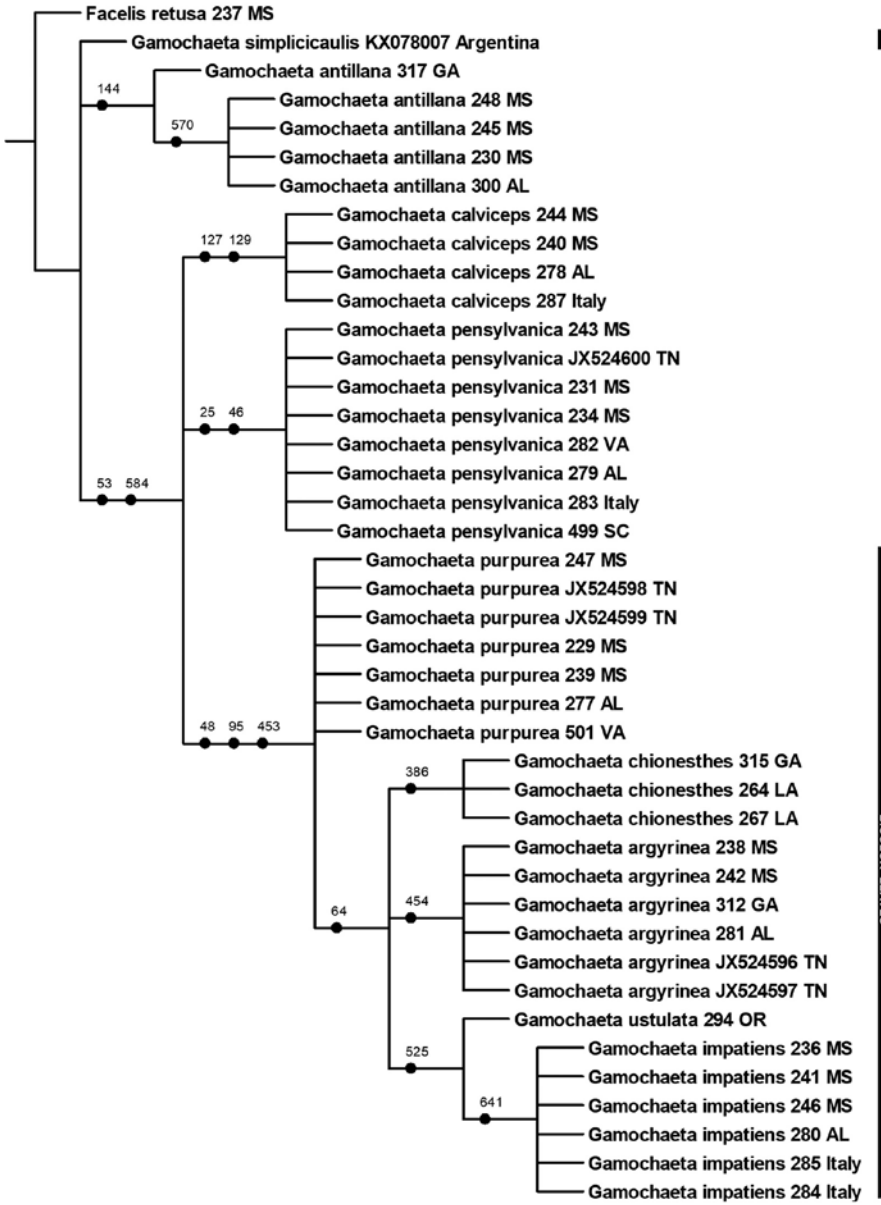

Figure 1. Most parsimonious tree (with unsupported nodes collapsed) of an analysis of nuclear internal transcribed spacer (ITS) data. Numbers on the branches refer to positions in the aligned data, with number 655 referring to the single multi-base-pair gap character (positions 453–456). Length = 17, ensemble consistency index = ensemble retention index = 1.00. Following each species name is a lab reference number to a collection (see Appendix) and the abbreviated state or country from which it was collected. The vertical bar indicates the species that have bicolor leaves (i.e., adaxial surface glabrous to sparsely hairy and generally green and abaxial surface with dense indumentum obscuring the epidermis, generally white to gray).

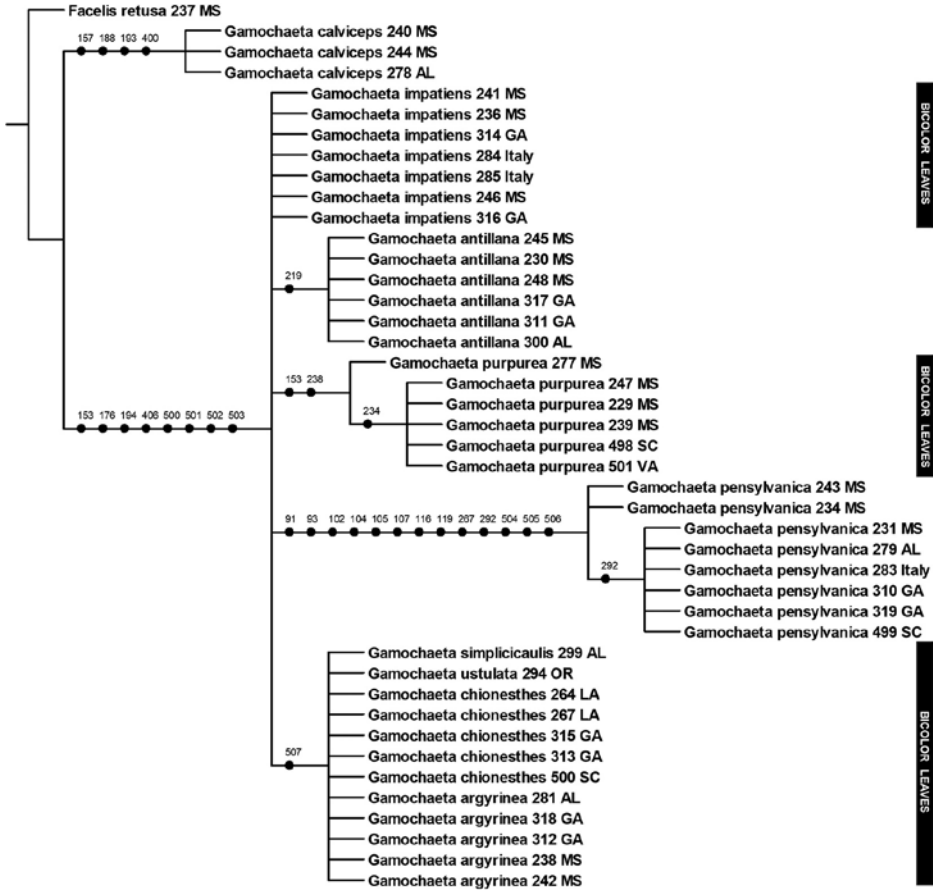

Figure 2. Most parsimonious tree (with unsupported nodes collapsed) of an analysis of plastid *trnH-psbA* intergenic spacer data. Numbers on the tree refer to positions in the aligned data, with numbers 500, 501, 502, 503, 504, 505, 506, and 507 referring to the gap characters at positions 52–56, 229–234, 285–290, 314–318, 90, 118, 410–419, and 339–360, respectively. Length = 31, ensemble consistency index = ensemble retention index = 1.00. Following each species name is a lab reference number to a collection (see Appendix) and the abbreviated state or country from which it was collected. The vertical bars indicate the species that have bicolor leaves (i.e., adaxial surface glabrous to sparsely hairy and generally green and abaxial surface with dense indumentum obscuring the epidermis, generally white to gray).

data also recovered a clade of most of the species that have bicolor leaves (*G. argyrinea*, *G. chionesthes*, *G. impatiens*, *G. purpurea*, *G. ustulata*) supported by three synapomorphies (Figure 1); only *G. simplicicaulis*, which also has bicolor leaves, was not recovered in that clade. Contrary to Nesom's (2004a, b) hypothesis that *G. ustulata* is the closest relative of *G. argyrinea*, the results here place *G. ustulata* sister to *G. impatiens*. The results from the ITS data also include specimens from Virginia (sent by T. Wieboldt; see Weakley et al. 2012: 335) and Italy (sent by A. Soldano), which were suspected to be new introductions

in those areas. As can be seen in the figures, the identifications based on morphology also corresponded to the groups recovered with DNA data.

The plastid *trnH-psbA* data did not resolve all of the recognized species of *Gamochaeta* in the eastern United States but were still useful in distinguishing five species and a clade that consists of four others (one being the western *G. ustulata*). The plastid data were particularly useful for recognition of *G. calviceps* and *G. pensylvanica*, which have (at least) 12 and 13 character state differences from the other species, respectively. However, eight of those differences in *G. pensylvanica* are due to an inversion at positions 90–118, which could be considered a single difference. The relationships recovered in the phylogenetic analysis of the plastid DNA data (Figure 2) were not congruent with the relationships recovered from the nuclear DNA data (Figure 1). Many factors may be at play (e.g., concerted evolution, plastid capture, hybridization). Although phylogenetic inference may require additional work, the DNA markers do provide suitable data for distinguishing species and for their identification using DNA barcodes.

Although DNA barcoding in plants has not achieved the success it has in animals (see Bolson et al. 2015 and references therein), DNA markers work better for identification in some groups of plants than others. Unlike other genera of the Compositae (e.g., *Solidago* L., cf. Fazekas et al. 2008, 2009; Kress et al. 2005) that possess little genetic differentiation in commonly used barcoding regions of DNA, the species of *Gamochaeta* in the eastern United States, both native and introduced, can be identified using a single nuclear marker (ITS), and several may be identified with a single plastid marker (*trnH-psbA*; see also Schilling and Floden 2012). Given that species of *Gamochaeta* are naturalizing in many places on Earth, these data will provide an important resource for identification.

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DEDICATION

This paper is dedicated to Dr. Robert L. Wilbur, for whom this issue of *Rhodora* has been organized. The senior author (MHA) was a master's student of Dr. Wilbur, and Dr. Wilbur encouraged him to work through the papers of Nesom and annotate the

specimens of *Gamochaeta* in the DUKE herbarium in the late 1990s. Given Dr. Wilbur's propensity to collect weedy and ruderal species, DUKE probably has one of the best, if not the best, collections of *Gamochaeta* in the United States. Not surprisingly, Dr. Wilbur also collected some of the first naturalizations of *Gamochaeta* in Hawaii while posted there shortly after World War II (Alford 2012). His large number of collections certainly accelerated understanding of the variation in the genus and led to the initiation of this project.

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APPENDIX

Vouchers and GenBank accession numbers for the samples represented in this paper, organized in the following order: Species—Lab Number (noted in Figures 1 and 2) or GB (for sequences obtained from GenBank), state or country (outside the United States), voucher (herbarium), nuclear ITS GenBank accession number, and plastid *trnH-psbA* GenBank accession number. A dash (———) indicates that no data were collected or available for that marker.

Facelis retusa (Lam.) Sch. Bip.—237, Mississippi, *Alford 3897* (USMS), OP683837, OP763517.

Gamochaeta antillana (Urb.) Anderb.—230, Mississippi, *Alford 4155* (USMS), OP683847, OP763542; 245, Mississippi, *Alford 3892* (USMS), OP683846, OP763541; 248, Mississippi, *Brantley 006* (USMS), OP683845, OP763543; 300, Alabama, *Diamond 21249* (TROY), OP683848, OP763546; 311, Georgia, *Carter 20835* (USMS), ———, OP763545; 317, Georgia, *Carter 20839* (USMS), OP683849, OP763544.

Gamochaeta argyrinea G.L. Nesom—GB, Tennessee, *Schilling 07-2766* (TENN), JX524596, ———; GB, Tennessee, *Phillippe 35455*, JX524597, ———; 238, Mississippi, *Alford 3922* (USMS), OP683863, OP763536; 242, Mississippi, *Alford 3913* (USMS), OP683864, OP763537; 281, Alabama, *Alford 4227* (USMS), OP683866, OP763533; 312, Georgia, *Carter 20836* (USMS), OP683865, OP763535; 318, Georgia, *Carter & Carter 20827* (USMS), ———, OP763534.

Gamochaeta calviceps (Fernald) Cabrera—240, Mississippi, *Alford 3915* (USMS), OP683851, OP763538; 244, Mississippi, *Alford 3900* (USMS), OP683850, OP763539; 278, Alabama, *Alford 4223* (USMS), OP683852, OP763540; 287, Italy, *Marchetti 10554* (Herbarium Soldano), OP683853, ———.

Gamochaeta chionesthes G.L. Nesom—264, Louisiana, *Nesom 2010-147* (USMS), OP683860, OP763528; 267, Louisiana, *Nesom 2010-147* (USMS), OP683861, OP763529; 313, Georgia, *Carter 20828* (USMS), ———, OP763531; 315, Georgia, *Carter & Carter 20878* (USMS), OP683859, OP763530; 500, South Carolina, *Strong & Kelloff 5427* (USMS), ———, OP763532.

Gamochaeta impatiens G.L. Nesom (formerly treated as *G. coarctata* [Nesom 2006] or *G. americana* [Freire et al. 2021])—236, Mississippi, *Alford 3895* (USMS), OP683867, OP763554; 241, Mississippi, *Alford 3805* (USMS), OP683868, OP763553; 246, Mississippi, *Alford s.n.* (USMS), OP683869, OP763558; 280, Alabama, *Alford 4225* (USMS), OP683870, ———; 284, Italy, *Marchetti 10556* (Herbarium Soldano), OP683872, OP763556; 285, Italy, *Cibel s.n.* (MSNM), OP683871, OP763557; 314, Georgia, *Carter & Carter 20848* (USMS), ———, OP763555; 316, Georgia, *Carter 20841* (USMS), ———, OP763559.

Gamochaeta pensylvanica (Willd.) Cabrera—GB, Tennessee, *Browne 78* (TENN), JX524600, ———; 231, Mississippi, *Alford 4156* (USMS), OP683839, OP763522; 234, Mississippi, *Alford 3893* (USMS), OP683840, OP763521; 243, Mississippi, *Alford 3905* (USMS), OP683838, OP763520; 279, Alabama, *Alford 4224* (USMS), OP683842, OP763523; 282, Virginia, *Estienne s.n.* (VPI) (specimen noted in Weakley et al., 2012:335), OP683841, ———; 283, Italy, *Marchetti 14999* (Herbarium Soldano), OP683843, OP763524; 310, Georgia, *Carter 20834* (USMS), ———, OP763525; 319, Georgia, *Carter 20813* (USMS), ———, OP763526; 499, South Carolina, *Kelloff & Strong 1670* (USMS), OP683844, OP763527.

Gamochaeta purpurea (L.) Cabrera—GB, Tennessee, *Estes 7859* (TENN), JX524598, ———; GB, Tennessee, *Bresowar 122* (TENN), JX524599, ———; 229, Mississippi, *Alford 4154*

(USMS), OP683855, OP763548; 239, Mississippi, *Alford 3906* (USMS), OP683856, OP763549; 247, Mississippi, *Brantley 001* (USMS), OP683854, OP763547; 277, Alabama, *Alford 4222* (USMS), OP683857, OP763550; 498, South Carolina, *Strong & Kelloff 5434* (USMS), ———, OP763551; 501, Virginia, *Strong & Kelloff 5192* (US), OP683858, OP763552.

Gamochaeta simplicicaulis (Willd. ex Spreng.) Cabrera, GB, Argentina, *Zuloaga et al. 11702* (SI), KX078007, ———; 299, Alabama, *Diamond 16584* (TROY), ———, OP763518.

Gamochaeta ustulata (Nutt.) Holub—294, Oregon, *Straub 201* (USMS), OP683862, OP763519.