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Author: Panero, Jose L.

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GENERIC RELATIONSHIPS IN GOCHNATIOIDEAE (ASTERACEAE) INCLUDING *TEHUASCA*, A NEW GENUS FROM NORTHEASTERN MEXICO.

Jose L. Panero

Department of Integrative Biology, The University of Texas, 1 University Station C0930, Austin, TX 78712, U.S.A.

Author for correspondence (panero@utexas.edu)

Abstract: Phylogenetic analysis of the Internal and External Transcribed Spacer regions of nuclear ribosomal DNA was used to elucidate the taxonomic limits of the genus *Gochnatia* and generic relationships in Gochnatioideae. These analyses place *Richterago* and *Cnicothamnus* sister to *Gochnatia* and collectively sister to the discoid genera *Anastraphia* and *Nahuatlea*. *Gochnatia* is monophyletic and has three main clades each corresponding to formal sections of the genus, namely *Gochnatia*, *Moquiniastrium*, and *Pentaphorus*. The recently described genus *Nahuatlea* was not monophyletic with its species found in a grade of three clades and only one species sister to the Caribbean genus *Anastraphia*. *Nahuatlea magna* is sister to *Anastraphia* and a new genus, *Tehuasca*, is erected with this species as its type. *Tehuasca* shares with *Anastraphia* corollas with glandular trichomes but differs from *Anastraphia* in having white to yellow corollas, recurved lobes of the corollas and leaf margins without spiny mucros. *Tehuasca magna* is a native species of northeastern Mexico.

Keywords: *Anastraphia*, *Gochnatia*, *Nahuatlea*, long distance dispersal, Caribbean biogeography, Mexican Asteraceae

Subfamily Gochnatioideae was described (Panero & Funk, 2002) to include four genera of Mutisieae sensu Cabrera (1977), *Cnicothamnus*, *Cyclolepis*, *Gochnatia*, and *Richterago*, that form a well-supported clade in phylogenetic analysis of chloroplast DNA sequence data (Panero & Funk, 2008). There are no morphological synapomorphies for the subfamily; characters that have been used to define a *Gochnatia* complex (Freire et al., 2002) are also found in closely related taxa outside Gochnatioideae since the two Southeast Asian species of *Gochnatia* section *Leucomeris* were found to be sister to the genus *Nouelia* tribe Hyalideae of southwestern China (Panero and Funk, 2008). Sancho and Freire (2009) point out that genera of Gochnatioideae can be recognized by a combination of three characters including dorsally glabrous style branches, apiculate apical anther appendages and deeply dissected 5-lobed corollas. Except for *Cnicothamnus* and three species of *Richterago* (Roque & Pirani, 2014) members of the subfamily have discoid capitula. Gochnatioideae contains approximately 90

species distributed in South America, the Caribbean and Mexico.

A stable generic classification of the Gochnatioideae has not been reached mainly because of the difficulty in circumscribing the limits of the genus *Gochnatia*. Cladistic analyses of morphological characters show that *Gochnatia* as circumscribed by Cabrera (1971) is a polyphyletic genus (Bremer, 1994; Jiménez Rodríguez et al., 2004; Ventosa-Rodríguez and Herrera-Olivier, 2011a). Cabrera recognized six sections in the genus including *Discoseris*, *Gochnatia*, *Hedraiophyllum*, *Leucomeris*, *Moquiniastrium*, and *Pentaphorus*. Freire et al. (2002) synonymized section *Moquiniastrium* under sect *Hedraiophyllum* and recognized three additional sections, namely *Anastraphioides*, *Glomerata* and *Rotundifolia*. The process of defining a natural *Gochnatia* started with the removal of species of *Gochnatia* section *Discoseris* and placing them in *Richterago* (Roque & Pirani, 2001). Hind (2007) recognized the two species of section *Pentaphorus* at the genus level but Telleria et al. (2013) did not find pollen characters to differentiate it

from other South American gochnatias. *Anastraphia* was recently resurrected (Ventosa-Rodriguez & Herrera-Olivier, 2011b) to accommodate all the Caribbean species of *Gochnatia* placed by Freire et al. (2002) in section *Anastraphioides*. A phylogenetic study using nrITS concatenated with several chloroplast markers (Funk et al., 2014) shows that *Gochnatia* s. l. is paraphyletic with the South American species placed in two clades, one with members of *Gochnatia* section *Moquiniastrium* sister to *Richterago*, and the other clade including taxa of *Gochnatia* sections *Pentaphorus* and *Gochnatia* and sister to all other species of the subfamily except *Cyclolepis*. The Mexican species of *Gochnatia* that were strongly supported as sister to *Anastraphia* have been recognized subsequently as a new genus, *Nahuatlea* (Funk et al., 2017). Despite a lack of significant statistical support for the relationships of the three clades of South American *Gochnatia* representing sections *Gochnatia*, *Moquiniastrium* and *Pentaphorus*, Funk et al. (2014) choose to recognize these three groups at the genus level.

The phylogenetic relationships of *Cyclolepis* to other Gochnatioideae and Wunderlichioideae in nuclear genomic studies of Asteraceae (Mandel et al., 2019) is equivocal, and conflicts with earlier Asteraceae phylogenies based on plastid data (Panero & Funk, 2008; Panero et al., 2014). In concatenated analysis of nuclear genomic data, a *Cyclolepis*–Wunderlichioideae clade is moderately supported as sister to Gochnatioideae. In coalescent-based species tree estimation, *Cyclolepis* is sister to Gochnatioideae but these are collectively sister to Wunderlichioideae rather than a Hecastocleidoideae–Asteroideae clade as reconstructed from chloroplast DNA.

To ascertain generic relationships in Gochnatioideae I undertook a sequence analysis of ITS and ETS regions of the nuclear ribosomal DNA. In particular, I aimed to (1) evaluate the monophyly of the South American *Gochnatia* and (2) determine the relationships of *Nahuatlea* to other genera of Gochnatioideae.

MATERIALS AND METHODS

TAXONOMIC SAMPLING. Sampling was informed by previous molecular studies (Panero & Funk, 2008; Funk et al., 2014) and the taxonomic revision of *Gochnatia* (Cabrera 1971, Freire et al., 2002). Nomenclatural types for all sections of *Gochnatia* were included except for those of sect *Rotundifolia* and sect. *Hedraiophyllum* that were not available. All species of *Nahuatlea* were sampled. Exemplars of six genera from the basal grade of Asteraceae clades outside Gochnatioideae were also included: *Stiffitia* (Stifftioideae), *Hyalis*, *Ianthopappus*, *Leucomeris*, *Nouelia* of the Hyalideae, and *Wunderlichia* (Wunderlichieae, Wunderlichioideae). The genus *Plazia* of Mutisioideae served as outgroup. A list of the 40 species, including 33 Gochnatioideae, analyzed in the study is provided in Appendix 1.

MOLECULAR METHODS. Total genomic DNA was isolated from field-collected leaves preserved in liquid nitrogen, CTAB solution or silica using the CTAB method (Doyle and Doyle 1987) modified to include a one volume phenol-chloroform-isoamyl alcohol extraction and two 70% ethanol washes. Total DNA was resuspended in water - 7 M sodium acetate (10:1), precipitating the DNA with one volume of ethanol, followed by two ethanol washes. Herbarium and some silica-preserved samples were isolated using the DNeasy® Plant Mini Kit from Qiagen, Inc. (Qiagen Sciences, Germantown, MD, USA).

DNA fragments were amplified using the polymerase chain reaction (PCR) in 50 µl reactions with 2 units of Taq polymerase, 0.2 M Tris-HCl, 8 mM (NH₄)₂SO₄, 0.2 mM dNTPs, 5 mM MgCl₂, 20 µM of primers, target DNA and water to a volume of 50 µl. PCR amplification protocols for the ITS and ETS included the following steps: 95 °C for 4 min, 50 °C for 45 s, 72 °C for 1 min followed by 36 cycles of 95 °C for 1 min, 50 °C for 45 s, 72 °C for 1 min with a 2 s extension per cycle and a final extension of 72 °C for 8 min (Rivera et al., 2016). Primers 18S-ETS (Baldwin and Markos, 1998) and a newly

developed primer ETS-Goch (5' GAT GTC TGC TTG CGC AGC AAC G-3') were used to amplify and sequence the ETS region. The ETS-Goch primer was developed to amplify the ETS region of the nuclear ribosomal IGS for the basal lineages of the Asteraceae. The ETS primer in the IGS region developed for tribe Madieae of the Heliantheae alliance (Baldwin and Markos 1998) did not amplify a majority of samples of subfamilies Barnadesioideae, Gochnatioideae, Mutisioideae, Stiffioideae, or Wunderlichioideae. The ETS primer developed for tribe Cardueae (Kelch and Baldwin, 2003) was used instead but this only amplified a very weak product for the genus *Cnicothamnus*. The *Cnicothamnus* ETS fragment was cloned using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA) and sequenced with the plasmid primers T7 and M13R to obtain the ETS primer sequence for *Cnicothamnus*. ETS-Goch was used to amplify most sequences included in this study. The ITS region was amplified using primers ITS 5 and ITS 4 of White et al. (1990) and occasionally primer ITS 7.5 (GAGTCATCAGCTCGCGTTGAC TA; Plovanich and Panero, 2004) in lieu of ITS 5 in amplification and sequencing reactions. Genbank accession numbers for the resulting sequences are provided in Appendix 1.

Sequences were edited using Sequencher v 4.9 (Gene Codes Corporation, Ann Arbor, USA) and assembled into primer-based matrices. Matrices were exported as Nexus interleaved files and concatenated in Paup* 4.0a build 163 (Swofford, 2002) then imported into Mesquite v 3.04 (Maddison and Maddison, 2015) to produce nbrf files. Alignment of the nbrf-formatted files was performed at the European Bioinformatics Institute website (<http://www.ebi.ac.uk/Tools/msa/mafft>) using MAFFT (Katoh and Standley, 2013). The aligned matrices were imported into Mesquite, edited for minor changes in homology and subsequently exported as simplified Nexus interleaved files and concatenated in Paup* v. 4.0a, build 166. The concatenated matrix was exported as a simplified Nexus sequential file to use in phylogenetic analyses.

PHYLOGENETIC ANALYSES. Bayesian phylogenetic analyses were performed for the ITS plus ETS concatenated dataset. Models of molecular evolution were evaluated under the Akaike Information Criteria (Akaike, 1974) using the program MrModeltest 2.1 (Nylander, 2004). Number of informative characters was calculated by PAUP* (Swofford, 2002). The concatenated dataset was treated as a single partition with the model of evolution chosen using MrModeltest 2.1 specified as GTR + G + I. Two independent runs of four Markov chains, each starting with a random tree for 40 million generations was implemented, sampling trees at every 5,000th generation. Stationarity of the chains was ascertained using ESS (Effective Sample Size) values above 200 as viewed in Tracer v1.7.1 (Rambaut et al., 2018) and Mr. Bayes 3.2.6 (Ronquist et al., 2012). The first 10% of the trees were discarded as burn-in samples. The 50% majority rule consensus tree was calculated by MrBayes 3.2.6 (Ronquist et al., 2012). Analyses were performed in the Cyberinfrastructure for Phylogenetic Research (CIPRES) cluster (Miller et al., 2015, <https://www.phylo.org>). A maximum clade credibility (MCC) tree was constructed in TreeAnnotator v1.10 (Rambaut et al., 2018) depicting the maximum sum of posterior clade probabilities and visualized and edited in FigTree v1.4.3 (Rambaut et al., 2018, <http://tree.bio.ed.ac.uk/software/figtree/>).

Corolla and trichome morphology was observed and recorded under polarized light using a Nikon Eclipse Ni compound light microscope at the microscopy and imaging facility of the University of Texas.

RESULTS

The aligned ITS and ETS matrix was 1079 base pair (bp) long (669 bp for ITS, and 410 bp for ETS) of which 575 characters were constant and 378 of these were parsimony-informative.

Gochnatioideae was polyphyletic because *Cyclolepis* was sister to all other genera of the subfamily and *Wunderlichia* (Wunderlichioideae), but statistical support was

below a 0.99 significance threshold (95–98%, Fig. 1). All genera of Gochnatioideae were monophyletic except for *Nahuatlea*. *Nahuatlea* was supported in a clade with *Anastraphia*, but *N. magna* alone was strongly supported as sister to *Anastraphia*. *Nahuatlea hypoleuca*, the type species, formed a clade with three other nahuatleas, that with a well-supported *N. arborescens* + *N. smithii* clade formed a grade (Fig. 1). *Cnicothamnus* was supported in a clade with *Gochnatia*, *Richterago*+*Nahuatlea* (including *N. magna*), and *Anastraphia*, but its position sister to *Richterago* and collectively sister to *Gochnatia* resolved with less than 95% statistical support.

DISCUSSION

Phylogenetic analysis of nrITS+ETS (Fig. 1) provided strong evidence to support recognition of a monophyletic *Gochnatia* that includes three of the six sections of the genus recognized by Cabrera (1971), namely sections *Gochnatia*, *Moquiniastrium* (including *G. cordata*) and *Pentaphorus*, and *G. palosanto* of section *Hedraiophyllum*. This novel concept excludes the Caribbean and Mexican species of *Gochnatia* that have been recently transferred to the genera *Anastraphia* and *Nahuatlea*, respectively (Ventosa-Rodriguez and Herrera-Oliver 2011b; Funk et al., 2014; 2017). An alternate taxonomic view that recognizes *Moquiniastrium* and *Pentaphorus* at generic rank is similarly consistent with the ITS+ETS phylogeny. However, the paraphyletic relationship found in Funk et al. (2014) lacks significant statistical support, thus there is no phylogenetic impediment to accepting these in *Gochnatia*. *Gochnatia* as here circumscribed can be separated from other Gochnatioideae by a distinctive combination of morphological characters including discoid capitula, a shrubby habit, and curled or reflexed corolla lobes.

The genus *Nahuatlea*, is currently circumscribed is polyphyletic in the ITS+ETS phylogeny. *Nahuatlea magna* was strongly supported as sister to *Anastraphia* and herein recognized as a new monospecific

genus, *Tehuasca* (*Tehuasca magna* in Fig. 1). There is evidence for the *Nahuatlea arborescens* + *N. smithii* clade outside the type-containing *Nahuatlea* clade but its phylogenetic position is not statistically supported so taxonomic assessment is equivocal. *Tehuasca magna*, a small shrub or small tree with broadly ovate leaves from central-northeastern Mexico, shares with most *Anastraphia* species pubescent corollas as opposed to glabrous as in nahuatleas (Fig. 2). Whereas most species of *Anastraphia* have well-developed spiny mucros, *Tehuasca* has entire margins.

Excluding the South American monotypic genus *Cyclolepis* from Gochnatioideae, the ITS+ETS phylogeny shows two sister lineages of the subfamily (Fig. 1) each of which contains genera that are geographically confined to either North or South America. *Anastraphia* and species originally described in *Nahuatlea* are North American/Caribbean, whereas *Gochnatia*, *Richterago* and *Cnicothamnus* are South American endemics (Fig. 1). *Cyclolepis*, Wunderlichioideae, as well as more basal Asteraceae are South American in origin. Based on our phylogeny, the North American lineage is, probably, the result of long distance from South America. Diversification and dispersal in Mexico ultimately resulted in later spread to the Caribbean and allopatric speciation of *Anastraphia* on multiple islands.

Nahuatlea magna is recognized as a new, monospecific genus, *Tehuasca*. Of all the species of North American Gochnatioideae, *Tehuasca magna* is the most hygrophytic with populations of the species found in tropical, seasonally dry, xerophytic to moist forests.

***Tehuasca* Panero, gen. nov.** Type: *Tehuasca magna* (M. C. Johnst. ex Cabrera) Panero

Similar to *Anastraphia* in having deeply dissected corollas and globular glandular trichomes on the abaxial surface of corollas but differs in having leaves with entire margins, coiled corolla lobes, and mostly creamy white, sometimes pale yellow, corollas.

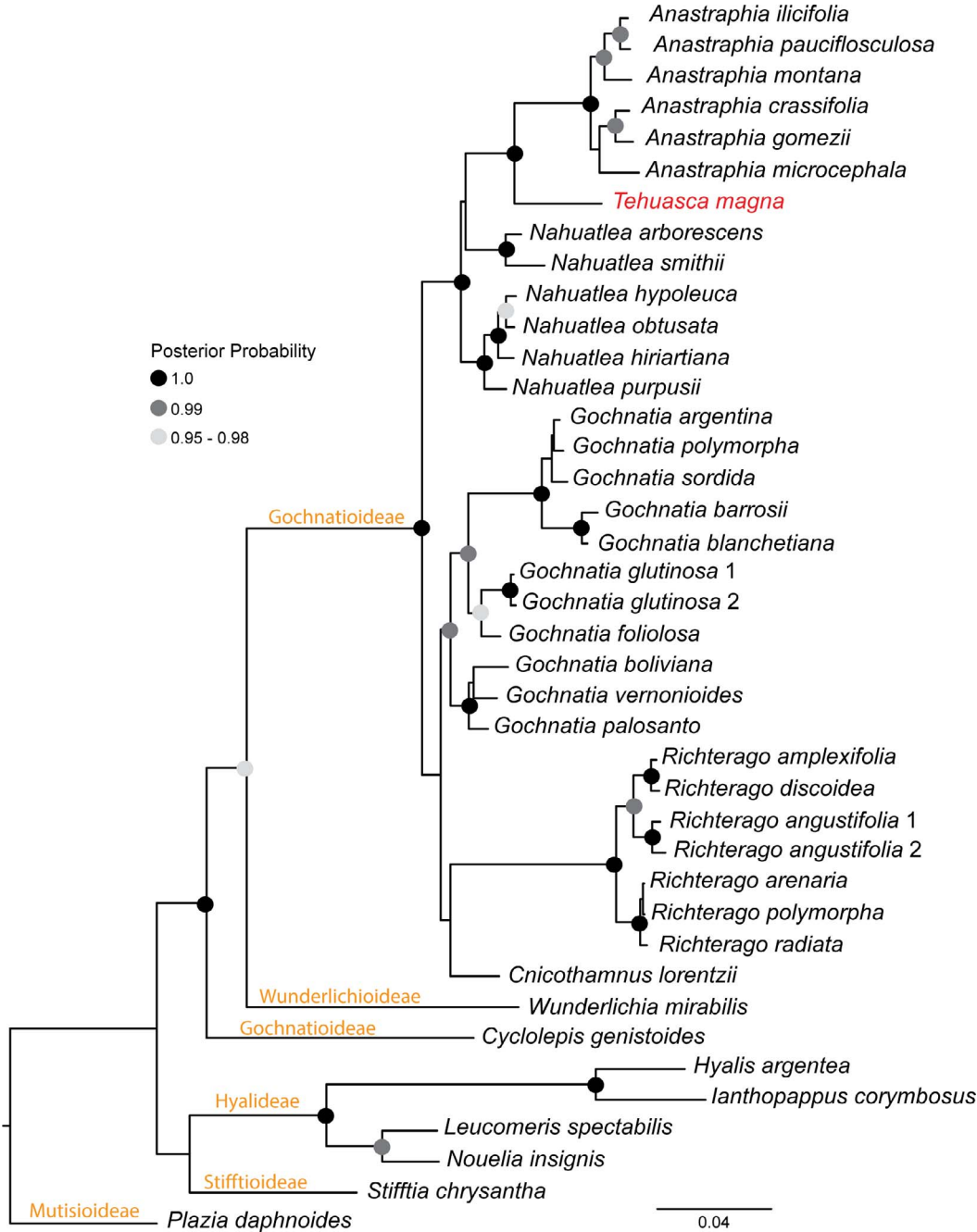


FIG. 1. Phylogenetic relationships among genera of Gochnatioideae based on Bayesian analysis of nuclear ribosomal internal and external transcribed spacer DNA sequences. Subfamily or tribal names label branches leading to the most recent common ancestor.

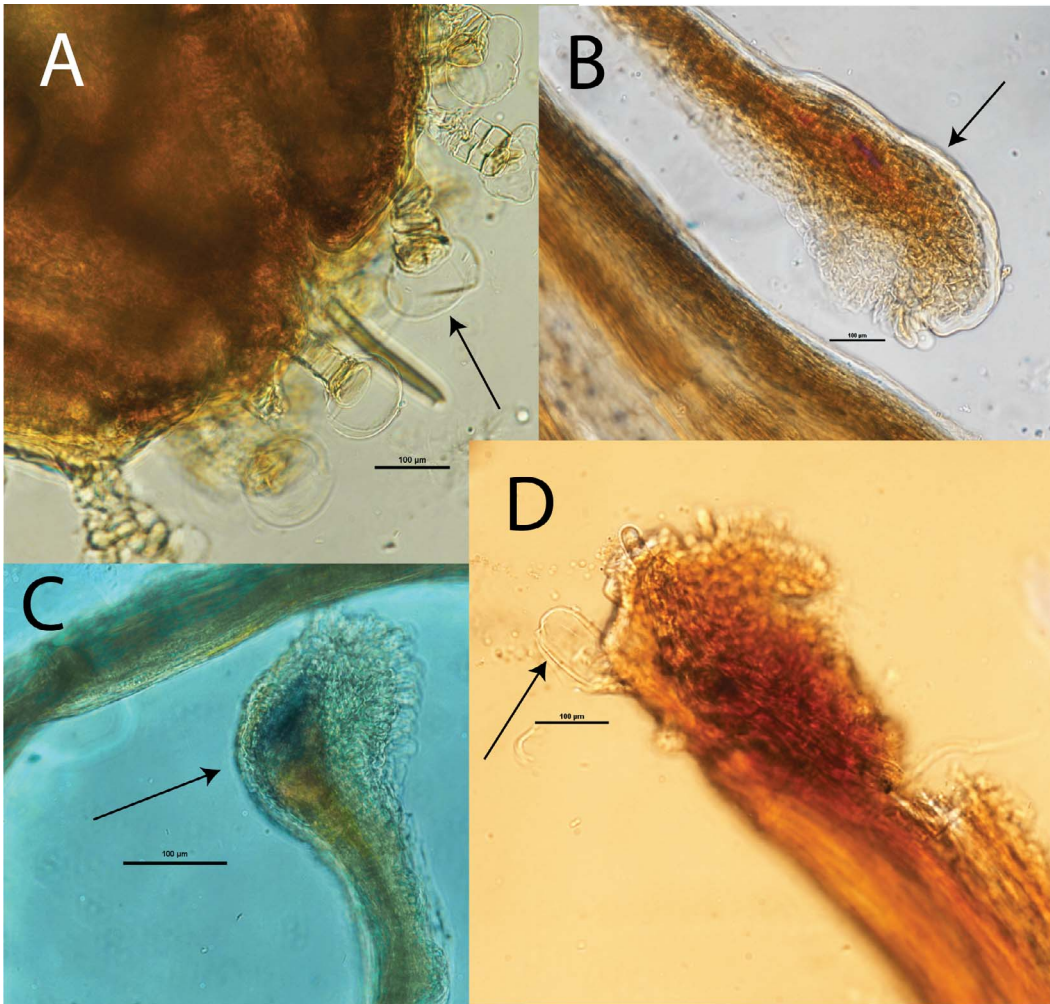


FIG. 2. Polarized light micrographs of corollas of representative species of North American and Caribbean Gochnatioideae. A. *Tehuasca magna* (Servin 1457, TEX). Arrow points to biseriate glandular trichomes at distal ends of corolla lobes. B. *Nahuatlea hypoleuca* (G. B. Hinton 18096, TEX). Arrow points to glabrous abaxial surface of corolla lobe. C. *Nahuatlea smithii* (Ortega 121, TEX). Arrow points to glabrous abaxial surface of corolla lobe. D. *Anastraphia sessilis* (García & Pimentel 787, TEX). Arrow points to biseriate glandular trichomes at abaxial, distal end of corolla lobes.

Shrubs or small trees. Leaves alternate, simple, petiolate, blades ovate to ovate-obovate, rarely suborbicular, apices acute to acuminate, leaf margins entire. Capitulescences of simple cymes. Capitula discoid, involucre campanulate, phyllaries in 5-6 series, phyllaries imbricate. Disc flowers 40-80, creamy white or yellow, abaxial surface mostly glabrous except for the distal end of lobes that are covered with mostly biseriate

glandular trichomes, lobes coiled. Anthers five, white, with small tails and narrowly lanceolate appendages, endothelial cells with polar thickenings, cells of the appendages and distal third of anthers sclerified. Styles with short, acuminate lobes, yellow, glabrous. Cypselae densely sericeous, silvery, pappus of multiple, stramineous bristles of equivalent length, distal end of bristles slightly expanded.

Tehuasca is endemic to the tropical moist and deciduous forests of eastern central/northern Mexico in the states of Guanajuato, Hidalgo, Queretaro, San Luis Potosi, and Tamaulipas. The name *Tehuasca* is an anagram of Huasteca. A significant portion of the geographic range of *Tehuasca* is within the boundaries of the Huasteca or Huasteca Potosina region, the pre-Columbian home of the Huasteca people. The Huasteca is a region of unusual beauty whose numerous limestone mountains have been eroded by multiple surface and underground rivers.

Tehuasca magna (M. C. Johnst. ex Cabrera)

Panero, comb. nov. Basionym: *Gochnatia magna* M. C. Johnst. ex Cabrera, Revista Mus. La Plata, Secc. Bot., 12(66): 147-150. 1971. *Nahuatlea magna* (M. C. Johnst. ex Cabrera) V. A. Funk, Phytokeys 91: 112. TYPE: MEXICO. Tamaulipas: 0.5 miles E of Nuevo Morelos, Oct 25 1959, J. Graham and M. C. Johnston 4485 (HOLOTYPE: MEXU; ISOTYPE: TEX!)

Shrubs or small trees 2-5 m tall. Leaves alternate, simple, petiolate, petioles 0.4-12 mm long, blades ovate to ovate-obovate, rarely suborbicular, apices acute to acuminate, leaf margins entire, adaxial surface green, sparsely pubescent, abaxial surface creamy in color, densely pubescent, approximately 3-10 cm long, 1.5-7 cm wide. Capitulescences of simple cymes with 2-9 capitula. Capitula discoid, involucre campanulate to urceolate, 1.2-1.7 cm long, 1-1.5 cm wide, phyllaries in 5-6 series, phyllaries imbricate, densely pubescent on abaxial surfaces, pubescence gradually decreasing from base to tip, distal end of phyllary glabrescent to glabrous, apices acuminate ending in a small mucro. Disc flowers 40-80 creamy white or yellow, abaxial surface mostly glabrous except for the distal end of lobes that are covered with mostly biseriate glandular trichomes, the trichomes with broadly expanded, globular, terminal cells, lobes coiled. Anthers five, white, with small tails and narrowly lanceolate appendages, endothelial cells with polar thickenings, cells of the appendages and distal third of anthers

sclerified. Styles with short, acuminate lobes, yellow, lobes 0.4 mm long, glabrous. Cypselae moderately to densely sericeous, silvery brown, 3.5-4 mm long, pappus of multiple, stramineous bristles of equivalent length, distal end of bristles slightly expanded.

Tehuasca magna, as the species epithet implies, has large capitula arranged in simple cymes with multiple series of imbricate phyllaries. However, these capitula are not the largest of the North American Gochnatioideae. The relatively recently described *Nahuatlea hiriartiana* (González Medrano et al., 2004) from the desert region of Hidalgo state in central Mexico has the largest capitula of the group. Cabrera (1971) stated that *Tehuasca magna* shares several characteristics with *Gochnatia arborescens* including broadly ovate leaves and capitulescences with few capitula arranged in condense, simple cymes or glomerules, but that their capitula were quite different. According to Cabrera the involucre of *N. arborescens* is campanulate-turbinate but campanulate in *T. magna*, the phyllaries are arranged in 8-10 series (vs. 5-6 series in *T. magna*) and the number of flowers is quite different with *N. arborescens* having 13-20 flowers vs. 40-80 in *T. magna*.

KEY TO THE GENERA OF GOCHNATIOIDEAE

1. Plants of North America
 2. Plants endemic to the Caribbean. Leaf margins with spiny mucros..... **Anastrophia**
 2. Plants of Mexico and the USA. Leaf margins entire
 3. Apices of abaxial surface of corolla lobes without glandular trichomes..... **Nahuatlea**
 3. Apices of abaxial surface of corolla lobes with prominent glandular trichomes..... **Tehuasca**
1. Plants of South America
 4. Spiny shrubs. Longest bristles of pappus with plumose distal ends..... **Cyclolepis**
 4. Shrubs or perennial herbs, not spiny. Pappus various.
 5. Capitula radiate
 6. Corollas vermilion to bright orange; small trees or shrubs in moist forests of Andean northern Argentina and southern Bolivia..... **Cnicothamnus**

6. Corollas white, pink, rarely purple; perennial herbs commonly forming rosettes rarely with short, leafy shoots of central and southern Brazil..... **Richterago**
5. Capitula discoid (sometimes peripheral corollas of capitulum slightly zygomorphic in *Gochnatia*)
7. Shrubs or trees, sometimes glutinous **Gochnatia**
7. Perennial herbs **Richterago**

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APPENDIX 1. LIST OF SPECIMENS OF
ASTERACEAE AND OUTGROUP TAXA
SEQUENCED IN THIS STUDY. GENBANK
ACCESSION NUMBERS ARE AS FOLLOWS: ETS,
ITS, NS = NO SEQUENCE AVAILABLE.

Anaestrophia crassifolia Britton, MN457774, KF989534. *Anaestrophia gomezii* León, MN457773, KF989537. *Anaestrophia ilicifolia* D. Don, MN457775, 457813. *Anaestrophia microcephala* Griseb., MN457778, NS. *Anaestrophia montana* Britton, MN457779, NS. *Anaestrophia pauciflorescens* C. Wright ex. Hitchc., MN457776, MN457814. *Cnicothamnus lorentzii* Griseb., MN457761, MN457799. *Cyclolepis genistoides* D. Don, MN457791, MN457827. *Gochnatia argentina* (Cabrera) Cabrera, MN457764, MN457802. *Gochnatia barrosii* Cabrera, MN457765, MN457803. *Gochnatia blanchetiana* (DC.) Cabrera, MN457766, MN457804. *Gochnatia boliviana* S. F. Blake, MN457767, MN457805. *Gochnatia foliolosa* (D. Don) D. Don & Arn. ex Hook., MN457772, MN457810. *Gochnatia glutinosa* (D. Don) D. Don & Arn. Ex Hook., 1, MN457762, MN457800. *Gochnatia glutinosa* (D. Don) D. Don & Arn. Ex Hook., 2, MN457763, MN457801. *Gochnatia palosani*

MN457824. *Richterago polymorpha* (Less.) Roque, MN457789, MN457826. *Richterago radiata* (Vell.) Roque, MN457790, MN457826. *Stiffitia chrysantha* Mikan,

MN457798, MN457834. *Tehuasca magna* (M. C. Johnst. ex Cabrera) Panero, MN457777, MN457815. *Wunderlichia mirabilis* Riedel, MN457792, MN457828.