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Origins and recent radiation of Brazilian Eupatorieae (Asteraceae) in the eastern Cerrado and Atlantic Forest ☆

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ABSTRACT

The remarkable diversity of Eupatorieae in the Brazilian flora has received little study, despite the tribe's very high levels of endemism and importance in the threatened Cerrado and the Atlantic Forest biodiversity hotspots. Eupatorieae are one of the largest tribes in Asteraceae with 14 of 19 recognized subtribes occurring in Brazil. We constructed the largest phylogeny of Brazilian Eupatorieae to date that sampled the nrITS and ETS, chloroplast *ndh*I and *ndh*F genes, and the *ndh*I–*ndh*G intergenic spacer for 183 species representing 77 of the 85 Brazilian genera of the tribe. Maximum likelihood and Bayesian phylogenetic analyses showed that these species are not collectively monophyletic, so their distribution reflects multiple introductions into Brazil. A novel clade was found that includes 75% of the genera endemic to Brazil (Cerrado-Atlantic Forest Eupatorieae, "CAFE" clade). This radiation of at least 247 species concentrated in the Cerrado and Atlantic Forest biomes of central eastern Brazil is <7 my old and exhibits several ecologically diverse life forms. Eight subtribes of Brazilian Eupatorieae (Ageratinae, Alomiinae, Ayapaninae, Critoniinae, Disynaphiinae, Eupatoriinae, Gyptidinae and Hebecliniinae) and 16 genera (*Ageratum*, *Agrianthus*, *Austroeupatorium*, *Bejaranoa*, *Chromolaena*, *Critonia*, *Disynaphia*, *Grazielia*, *Hatschbachiella*, *Heterocondylus*, *Koanophyllon*, *Lasiolaena*, *Neocabreria*, *Praxelis*, *Stylotrichium*, and *Symphyopappus*) were found to be polyphyletic. We attribute incongruities between the molecular phylogenetic results and the current classification of the tribe mostly to convergent evolution of morphological characters traditionally used in the classification of the tribe. We used these phylogenetic results to suggest changes to the classification of some subtribes and genera of Eupatorieae that occur in Brazil.

1. Introduction

Despite the race to document the vegetation of the Brazilian Cerrado (Borges et al., 2015) through inventories (Mendonça et al., 2008; Forzza et al., 2012) and phylogenetic studies (Chacón et al., 2008; Simon et al., 2009; Antonelli et al. 2010; Roncal et al., 2011; Simon and Pennington, 2012; Drummond et al., 2012; Ribeiro et al., 2012; Koenen et al., 2013; Lohmann et al., 2013; Perret et al., 2013; Givnish et al., 2014; Hernández-Hernández et al., 2014), the evolutionary origins and patterns of diversification of one of the largest components, Asteraceae, have received little attention. The Cerrado is the world's most biologically diverse savanna (Klink and Machado, 2005; Forzza et al., 2012) and a biodiversity *hotspot* undergoing massive anthropogenic transformation threatening species that make the biome famous for its high endemism (Myers et al., 2000; Forzza et al., 2012). Endemism is prevalent among Brazilian Eupatorieae with 440 species (73%) and 40 genera (48%) endemic to the country (Nakajima et al., 2014). Eupatorieae comprise about 30% of the Asteraceae flora of Brazil (Barroso, 1991) with 608 species (Nakajima et al., 2014). Of the 375 species native to the Cerrado half are endemic (Nakajima et al., 2014). More than half (56.9%) of the genera of Cerrado Eupatorieae are strictly endemic, and many others are nearly endemic extending just into adjacent biomes (Nakajima et al., 2014). In contrast to these, a sizable number of endemic species belong to a few widespread genera that are also found in North America and/or the Andes, for example *Mikania* and *Chromolaena*. However, because information about phylogenetic relationships of Brazilian Eupatorieae is lacking, it's not clear how the diversity of Eupatorieae in the Cerrado was formed.

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Phylogenetic relationships among most genera and major lineages of Eupatorieae, as well as among the 2000–2500 species, are not yet resolved. Historically, the classification of the tribe has been dominated by a large genus *Eupatorium* comprising approximately 60% of the tribe's species diversity. Based on morphological comparisons, King and Robinson (1987) recognize a smaller genus *Eupatorium* with only 45 species restricted to the northern hemisphere. Hind and Robinson (2007) classify the species of Eupatorieae in 182 genera. We recognize three additional genera, *Eutrochium* and the more recently described *Paneroa* and *Zyzyura*, bringing the total to 185. Slightly modifying the classification of King and Robinson (1987) but without providing descriptions or listing genera under subtribe, Robinson et al. (2009) recognize 19 subtribes. Molecular phylogenies are consistent with the narrower circumscription of *Eupatorium* (Schilling et al., 1999; Schmidt and Schilling, 2000). However, the classification system of Eupatorieae culminating in Robinson et al. (2009) has been criticized by several authors, and not adopted widely in floristic treatments (e.g., McVaugh, 1984; Cabrera and Klein, 1989; Barroso, 1991; Zuloaga et al., 2008). To define the taxonomic limits of genera and aggregate these into subtribes King and Robinson (1987) rely primarily on characteristics of the involucral bracts and several floral microcharacters, supplemented with information from presence or absence of secondary metabolites, geographic distribution, and chromosome numbers. Their hypotheses of relationships are criticized as more phenetic than phylogenetic, so shared characters may reflect convergent evolution rather than common ancestry (Scott, 1985; Nesom, 1989; Bremer et al., 1994). Indeed, recent molecular phylogenetic studies of nuclear ITS and chloroplast *trn*L-F provide evidence that subtribes Eupatoriinae (Schmidt and Schilling, 2000; Tippery et al., 2014), Gyptidinae (Ferreira, 2010; Tippery et al., 2014), Disynaphiinae (Hattori, 2013) and Ayapaninae (Fernandes, 2014) as circumscribed by King and Robinson (1987) are polyphyletic, and subtribes Piqueriinae, and Alomiinae may be polyphyletic as well (Robinson et al., 2009). These studies also show that some genera of Disynaphiinae and Ayapaninae are not monophyletic and that the main characters used to circumscribe Disynaphiinae are homoplasious (Hattori, 2013; Fernandes, 2014). Although previous phylogenetic studies make it clear that the current classification system of tribe Eupatorieae needs revision in order to reflect its evolutionary history, the monophyly of several Eupatorieae subtribes has not yet been tested, and the reconstruction of generic relationships is still very incomplete with no more than 20% of the tribe's genera sampled in any study.

A robust generic phylogeny is prerequisite to understand the historical assembly of these Asteraceae in the Cerrado, and for comparative studies of those species adapted to that seasonally dry and fire prone environment. Brazilian Eupatorieae are terrestrial (rarely aquatic) herbs, subshrubs, shrubs, trees and vines and are easily recognized among other Asteraceae by their discoid heads, flowers with white, pink to reddish or lavender corollas, conspicuous and much exserted styles with enlarged appendages, usually opposite leaves, and cypselae with black walls (Hind and Robinson, 2007; Fig. 1). They are distributed in all Brazilian biomes, but most occur in the Cerrado (Nakajima et al., 2014) and many show specific adaptations particularly to drought and fire regimes. These are currently classified in 85 genera (Nakajima et al., 2014) and 14 subtribes (Robinson et al., 2009). We reconstructed the phylogeny of Brazilian Eupatorieae by sampling all subtribes and more than 90% of genera. The subtribal richness of Brazilian Eupatorieae means we were able to challenge the monophyly of several subtribes and genera proposed by King and Robinson (1987) that have not been previously tested in molecular studies. We present the most thorough assessment of Eupatorieae subtribal and generic circumscriptions to date that has many implications for future taxonomic realignments across the entire tribe. The phylogeny reveals multiple origins and a Cerrado-distinguished radiation of Eupatorieae in Brazil.

2. Materials and methods

Taxon and character sampling—we sampled broadly within as well as among Brazilian Eupatorieae genera, including the type genus of each subtribe and many genera that are monotypic, rare or have anomalous morphologies, as monotypic, rare and anomalous taxa might represent independent lineages (Panero and Funk, 2008). Most samples were field collected and DNA extracted from silica dried leaf, capitulum or seed material; a few were extracted from leaf or capitulum tissue removed from herbarium specimens deposited in the TEX, UB, NY, MICH, and HUEFS collections. *Galeana pratensis* and *Eutetras palmeri* served as outgroup based on the sister relationship of tribe Perityleae (Panero and Funk, 2008). The combined data matrix included 183 species and 81 genera of Eupatorieae plus the outgroups (Table S1). All 14 Eupatorieae subtribes that grow in Brazil, 77 Brazilian genera (90.5%) and 156 Brazilian species (26%) were sampled.

For each taxon we attempted to sequence the nuclear ribosomal internal and external spacer regions (ITS-1, ITS-2 and ETS), and chloroplast regions *ndh*I gene, the *ndh*I–*ndh*G intergenic spacer, and the *ndh*F gene including part of the spacer between *ndhF* and *ycf-1*. These markers have proven usefulness in phylogenetic studies of Eupatorieae (Schmidt and Schilling, 2000; Ito et al., 2000; Ferreira, 2010; Hattori, 2013; Fernandes, 2014) and Asteraceae (Kim and Jansen, 1995; Panero et al., 2014). The concatenated chloroplast matrix included 147 species whereas the combined nuclear matrix included 183 species. Table S1 provides Genbank numbers of all sequences by taxon.

DNA extraction, amplification and sequencing—extraction of DNA was performed using the Qiagen DNeasy Plant Mini Kit following the Quick-Start Protocol (Qiagen, Valencia CA). Some DNA samples that showed a dark color after extraction were further cleaned using the Nexttec 1-step Plant kit purification step (Nexttec, Germany).

Amplification and sequencing of the nuclear ETS were conducted using the Ast-1 primer (CGTAAAGGTGTGTGAGTGGTTT) modified from Markos and Baldwin (2001) and primer 18S-Alt (TGAGCCATTCGCAGTTTCACAGTC), a modified version of primer 18-S of Baldwin and Markos (1998). For the ITS the primers used were ITS-4 (TCCTCCGCTTATTGATATGC) and ITS-5 (GGAAGTAAAAGTCGTAACAAGG) (White et al., 1990). For some taxa the ITS amplification failed but the presence of DNA was detected when total DNA extraction run in agarose gel was performed. In these cases, amplification was carried out in two parts using primers ITS-5 and ITS-2 (GCTGCGTTCTTCATCGATGC), and ITS-3 (GCATCGATGAAGAACGCAGC) with ITS-4 (White et al., 1990). The *ndh*I gene and *ndh*I–*ndh*G intergenic spacer regions were amplified and sequenced using primers *ndh*GF (CCGACCCTAGAAAGACTAAAAG) and *ndh*Aexon2R (CGTCCCAACTTCTTTCACTG) (Panero and Crozier, 2003). The *ndh*F 52 to 1212 region was amplified and sequenced using the *ndh*F 52 (AGGTAAGATCCGGTGAATCGGAAA) and *ndh*F 1212 (GGTGGAATACCACAAAGA) primers (Jansen, 1992; Panero and Crozier, 2003). The *ndh*F 972 and *ndh*F 1587 regions were amplified using primers *ndh*F 607 (ACCAAGTT CAATGTTAGCGAGATTAGTC) and *ndh*F 972 (GTCTCAATTGGGTTATATGATG) and sequenced using *ndh*F 972 and *ndh*F 1587 (CCAACCCTTTCTTTCTATTCCG) primers (Jansen, 1992; Panero and Crozier, 2003). PCRs included 2 units of Taq polymerase, 0.2 M Tris-HCl, 8 mM $(NH_4)_2SO_4$, 0.2 mM dNTPs, 5 mM $MgCl_2$, 20 μM of primers, target DNA and water to a volume of 50 μl. PCR amplification protocols for all markers included the following steps: 95 °C