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A dated phylogeny of the papilionoid legume genus *Canavalia* reveals recent diversification by a pantropical liana lineage ☆

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ABSTRACT

Canavalia is a pantropical legume genus of lianas comprising approximately 60 species distributed in a wide range of habitats. In the last taxonomic revision, the genus was divided into four subgenera: *Canavalia* (Pantropical), *Catodonia* (Neotropical, excepting one species also found in the Old World), *Maunaloa* (Hawaiian), and *Wenderothia* (Neotropical). In this study, we reconstructed the phylogeny of *Canavalia* using a broad taxon sampling and analyses of nuclear (ETS and ITS) and plastid markers (*trnK*/*matK*). We evaluated the infrageneric classification of the genus and investigated its biogeographical history using molecular dating analyses and ancestral area reconstructions. The phylogenetic analyses resolved subgenus *Wenderothia* as monophyletic. Subgenus *Catodonia* needs to be recircumscribed and the relationships between subgenera *Canavalia* and *Maunaloa* remain unclear. *Canavalia* arose during the Miocene with a mean stem age estimate of 13.8 Ma and mean crown age estimate of 8.7 Ma, and most extant species evolved during the Pleistocene. Several climatic and geological events are chronologically coincident with the divergence of the major clades of *Canavalia* (glacial/interglacial periods, Andes uplift and the formation of Pebas and post-Pebas systems, closure of the Isthmus of Panama, and change in the direction of ocean currents). Ancestral area reconstructions for the early divergence of the genus are equivocal, although, some evidence suggests *Canavalia* originated in the wet forests of South America and achieved its current pantropical distribution through recent transoceanic dispersal. The evolution of *Canavalia* is better explained by a series of several processes than by discrete historical events.

1. Introduction

Diversification studies of plants using time-calibrated molecular phylogenies reveal a wide range of rate heterogeneity through time, among lineages, and among different geographical and ecological settings (Hughes et al., 2015). The patters of diversification in wet forests, seasonally dry forests, savannas and montane habitats differ in the timing of major radiations, net diversification rates, and species persistence over time (Pennington et al., 2004; Drummond, 2008; Simon et al., 2009; Hoorn et al., 2010; Särkinen et al., 2012; Simon and Pennington, 2012; Lohmann et al., 2013; Côrtes et al., 2015; Koenen et al., 2015). These highly variable explanations of the biodiversity among co-existing lineages suggest that unique historical events are not at play in shaping present day patterns of plant diversity.

The same might be true for explaining the diversity of individual lineages, such as that of the legume genus *Canavalia* Adans., the focus of our study. The pantropical genus *Canavalia* includes about 60 species of lianas in a wide range of habitats, including both wet and dry forests, savannas, and beach vegetation (Sauer, 1964; Aymard and Cuello, 1991). In addition to the lianescent habit, the genus is also characterized by trifoliolate leaves, a pseudoracemose inflorescence, and resupinate flowers with a bilabiate calyx where the lower (carinal) lip bears three small teeth and the upper

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(vexillary) lip two larger lobes (Sauer, 1964). Some species have buoyant and impermeable seeds, which can drift for long periods over great distances (Sauer, 1964). Some species are used for food, cover crops, green manure, as ornamentals, and for medicine, e.g., jack bean [*Canavalia ensiformis* (L.) DC.] and sword bean [*C. gladiata* (Jacq.) DC.], both unknown in natural habitats (Sauer, 1964; NAS, 1979; Ekanayake et al., 2007).

The four subgenera of *Canavalia* (Sauer, 1964) show some ecological and biogeographical trends. Subgenera *Catodonia* J.D. Sauer and *Wenderothia* (Schltdl.) J.D. Sauer are restricted to the New World, except for *C. bonariensis* Lindl., which occurs in South America and Africa. Subgenus *Catodonia* is mainly from rainforest habitats, although some species pioneer beach vegetation. Subgenus *Wenderothia* occupies wet and seasonally dry forests and savannas. Subgenus *Canavalia* has a pantropical distribution and most of its species occur in wet forests, although some also pioneer beach vegetation (Sauer, 1964). Subgenus *Maunaloa* J.D. Sauer is endemic to the Hawaiian Islands and occupies wet and dry habitats (Sauer, 1964).*Canavalia* belongs to the tribe Diocleae (Queiroz et al., 2015), one of the groups included in the Canavanine Accumulating Clade (or the non-protein aminoacid accumulating clade), which represents the largest radiation of papilionoid legumes (Wojciechowski et al., 2004; Cardoso et al., 2012, 2013; LPWG, 2013). Phylogenetic studies of *Canavalia* based on morphological or molecular data have resolved the genus as monophyletic (Queiroz et al., 2003, 2015; Varela et al., 2004; Vatanparast et al., 2011). Morphology-based phylogenetic analyses of the Diocleae recovered *Canavalia* in a clade along with *Camptosema* W.J. Hook. & Arn., *Cleobulia* Mart. ex Benth., and *Cratylia* Mart. ex Benth (Queiroz et al., 2003). However, the studies using molecular markers indicated that the genus is sister to a clade that includes the remaining Diocleae (Doyle and Doyle, 1993; Kajita et al., 2001; Varela et al., 2004; Queiroz et al., 2015). Insufficient taxon sampling precluded the evaluation of phylogenetic relationships among the *Canavalia* subgenera with confidence.

Using Eocene and Miocene fossils supposedly belonging to different subgenera of *Canavalia*, Sauer (1964) hypothesized that the evolution of the genus was already advanced in the early Tertiary and thus the genus would have diverged from other Phaseoloid legumes during the Cretaceous. Sauer also suggested that *Wenderothia* could be the most primitive subgenus from which subgenera *Canavalia* and *Catodonia* would have been independently originated, and that subgenus *Maunaloa* would be an offshoot of subgenus *Canavalia*. Thus, Sauer (1964) inferred the monophyly of subgenera *Catodonia* and *Maunaloa* and the paraphyly of subgenera *Wendetrothia* and *Canavalia*.

In this study, we conducted a time-calibrated molecular phylogenetic analysis of *Canavalia* using a broad taxonomic sampling that was designed to address the following questions: (1) Are the intuitive hypotheses of Sauer (1964) about the paraphyly of subgenera *Wenderothia* and *Canavalia*, and the monophyly of subgenera *Catodonia* and *Maunaloa* supported by a phylogenetic framework? (2) Where and when did *Canavalia* originate and diversify? (3) How many dispersal events occurred between the New and the Old World? (4) Are those dispersal events related to seed buoyancy?

2. Material and methods

2.1. Taxon sampling

The sampling includes 57 accessions of *Canavalia* corresponding to 47 of the 61 species currently recognized for the genus (17 of the 26 species of subg. *Canavalia*, 10/12 of subg. *Catodonia*, 3/6 of subg. *Maunaloa*, and 17/17 of subg. *Wenderothia*). Also sampled were five undescribed species (Snak, 2015; C. Snak, unpublished data). For *C. bonariensis* Lindl., *C. mattogrossensis* (Barb. Rodr.) Malme, and *C. villosa* Benth. we included two accessions that could represent different species based on distinct patterns of morphology and distribution. In this study, nine species of subg. *Canavalia* (*C. aurita* J.D. Sauer, *C. macrobotrys* Merr., *C. mollis* Wall. ex Wight & Arn., *C. papuana* Merr. & Perry, *C. raiateensis* J.W. Moore, *C. ramosii* J.D. Sauer, *C. regalis* Dunn, *C. sericea* A. Gray, *C. veillonii* I.C. Nielsen), two of subg. *Catodonia* (*C. mandibulata* J.D. Sauer, *C. sericophylla* Ducke), and three of subg. *Muanaloa* (*C. galeata* Gaudich., *C. kauaiensis* J.D. Sauer, *C. napaliensis* St. John) could not be sampled. *Cleobulia multiflora* Mart. ex Benth. and *Dioclea virgata* (Rich.) Amshoff were chosen as outgroups based on the phylogeny of Diocleae (Queiroz et al., 2015). A complete list of vouchers are associated with GenBank accessions (Table 1).

2.2. DNA extraction, amplification and sequencing

Total genomic DNA was extracted from silica gel-dried leaves using the 2× CTAB protocol of Doyle and Doyle (1987). For herbarium samples (ca. 60%), DNA was extracted using the DNeasy Plant Mini Kit (QIAGEN GmbH, Hilden, Germany). Three DNA regions were selected for this study: plastid *trnK/matK* (the *matK* gene and partial flanking *trnK* introns), ribosomal nuclear ETS (partial 5′ end of the 18S ribosomal RNA gene and part of the External Transcribed Spacer) and ITS [3′ end of nuclear ribosomal 18S, 5.8S and flanking Internal Transcribed Spacers 1 and 2, and the 5′ end of 26S] (Table 2). We chose these regions because they have provided excellent resolution at different taxonomic levels across different clades of papilionoids (de Queiroz et al., 2010; Queiroz and Lavin, 2011; Silva et al., 2012; Cardoso et al., 2012, 2013, 2015; Perez et al., 2013; Souza et al., 2013).

PCR reactions were performed using the TopTaq Master Mix Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's protocol, for a final volume of 10 μL. For herbarium samples, PCR reactions also included 2 μL of TBT-PAR [trealose, bovine serum albumin (BSA), polysorbate-20 (Tween-20)] (Samarakoon et al., 2013), and for ITS they also included 0.2 μL of DMSO 99.5% (dimethyl sulfoxide) in order to avoid secondary conformations (Table 2).

PCR products were cleaned using PEG 11% (Paithankar and Prasad, 1991), and then sequenced in both directions using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Austin, Texas, USA) according to the following protocol: a hot start followed by 3 min of initial denaturation at 96 °C, 30 cycles of 96 °C denaturation for 20 s, 50 °C annealing for 15 s, and 60 °C extension for 4 min. Sequencing products were cleaned using isopropanol 80% and ethanol 70%, and analyzed on a 3130*xl* Genetic Analyzer (Applied Biosystems/HITACHI, Tokyo, Japan) at the Laboratório de Sistemática Molecular de Plantas of the Universidade Estadual de Feira de Santana (LAMOL/UEFS).

2.3. Alignment and phylogenetic analyses

The electropherograms were assembled and edited using the Geneious platform (Drummond et al., 2012a). Multiple sequence alignment was performed using MUSCLE (Edgar, 2004) with default settings. Alignments were inspected and adjusted manually using Geneious (Drummond et al., 2012a). The alignments for each data set are available in TreeBase (submission 18801). Unambiguous indels were coded for all data sets using the simple indel coding criteria (Simmons and Ochoterena, 2000), as implemented in the Seqstate software (Müller, 2005a). We performed maximum parsimony (MP) and Bayesian analyses (BA) for individual, nuclear (concatenated ETS and ITS), and combined (all regions) data sets. Conflicts among data sets were evaluated by the incongruence