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A TIME-CALIBRATED PHYLOGENY OF *VERBESINA* (HELIANTHEAE – ASTERACEAE) BASED ON NUCLEAR RIBOSOMAL ITS AND ETS SEQUENCES

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Verbesina L. is a genus of the tribe Heliantheae, subtribe Verbesininae (Asteraceae), with distribution in the Americas, where Mexico and the Andes harbour the richest concentration of species. The approximately 325 species in the genus are shrubs, subshrubs, trees and rarely herbs. Despite its high species diversity and biogeographical importance, the only available phylogenetic hypothesis for *Verbesina* was based on chloroplast DNA restriction site data. In the present study, nuclear ITS and ETS DNA sequence data were used with an expanded taxon sampling, particularly among the South American *Verbesina* species, to improve phylogenetic resolution and support, clarify infrageneric relationships, and resolve biogeographical questions in the genus. The results of our new analysis corroborate the monophyly of *Verbesina*, but its current classification into 12 taxonomic sections, based on morphological characters such as phyllotaxis, head size, corolla colour, and presence of ray flowers, is not congruent with the molecular phylogeny, in which most sections are polyphyletic. We also show that *Verbesina* diverged in the late Miocene of North America, about 8 Ma. At least two independent Pleistocene dispersals into South America across the Isthmus of Panama and along the Southern Andes are evident, beginning around 3.23 Ma (1.27–3.23 Ma) in the Middle Pliocene, and resulting in an extra-Amazonian distribution of the genus in South America. Diversification in South America began around 2.83 Ma with occupation of the Andes. Colonisation of Brazil is estimated to have occurred around 2.15 Ma, from Andean lineages.

Keywords. Biogeography, dispersal, infrageneric classification, Neotropics
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Introduction

Verbesina L. is positioned in the subtribe Verbesininae of the tribe Heliantheae (Asteraceae), one of the 13 tribes that make up the Heliantheae Alliance, characterised by possession

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of black cypselae due to the presence of phytomelanin. *Verbesina* species are subshrubs, shrubs, small trees, and rarely herbs, with alternate or opposite, entire, or pinnatifid to pinnatipartite, corymboid capitulescence, and discoid or radiate capitula, with white, orange, red, yellow, greenish-white, rose or purplish-green corollas. The diagnostic feature of the genus is the strongly compressed, biconvex cypselae with blackish, smooth or verrucous surfaces, two, rarely three, evident entire or dentate lateral wings, and crowned by two, rarely three, erect awned pappi (**Figure 1**). The pollen grains are oblate-spheroidal to prolate-spheroidal, medium to large, isopolar, 3-colporate with a subtriangular amb, a small polar area, a long colpus, a lalongate endoaperture, a caveate exine and an echinate sexine (Gonçalves, 1976; Moreira *et al.*, 2018). The chromosome number for the majority of *Verbesina* species is *x* = 17, but *x* = 16 and x = 18 have also been reported (Anderberg *et al.*, 2007; Panero & Strother, 2021).

The genus comprises more than 325 species (Panero & Strother, 2021; POWO, 2023) distributed in the Americas, with their northern limit in Canada and reaching Argentina in the south (Panero, 2007). The species are a typical component of montane and premontane areas of humid or cloud forests, semi-deciduous seasonal forests, and gallery forests. Taxonomic revisions for *Verbesina* (e.g. Blake, 1925; Coleman, 1966a, 1966b; Olsen, 1985; Turner, 1985; Olsen, 1988; Turner, 2008) indicate that approximately two-thirds of the species are from North and Central America (including the Caribbean), while a third are from South America. Panero & Strother (2021) indicated that the greatest diversity of species is found in the highlands of Mexico and the Andes in humid or cloud forests, while most shrubby and perennial herbaceous species occur in open, dry areas of northern Mexico and southwestern USA. In South America, *Verbesina* is a component of highland tropical, seasonal semi-deciduous and gallery forests, occurring from the coastal lowlands to high elevations in the Atlantic Rain Forest biome. The genus is less well represented in open and dry biomes such as the Cerrado and Caatinga and is almost absent in the Amazon basin (Moreira & Cavalcanti, 2020).

Candolle (1836) proposed the first infrageneric classification for *Verbesina*, dividing it into three sections: *Verbesina* sect. *Verbesinaria* DC., including 29 species; *Verbesina* sect. *Hamulium* DC., with one species; and *Verbesina* sect. *Platypteris* DC., comprising 11 species. Gray (1884) added two more sections to this classification: *Verbesina* sect. *Pterophyton* A.Gray, with 14 species, and *Verbesina* L. sect. *Ximenesia* A.Gray, with two species.

Robinson & Greenman (1899) prepared a synopsis including 109 species of *Verbesina*, adding seven new sections and recognising 12 sections for the genus (**Supplementary file 1**). Based mainly on usually variable morphological characters, such as opposite or alternate phyllotaxy, inflorescence type, capitulum size, and the presence or absence of ray flowers, the sections have, with few exceptions, wide geographical boundaries, occurring in South, Central and North America. Only *Verbesina* sect. *Alatipes* B.L.Rob. & Green, *Verbesina* sect. *Platypteris*, *Verbesina* sect. *Petrophyton* A.Gray, and *Verbesina* sect. *Sonoricola* B.L.Rob. & Greenm. are

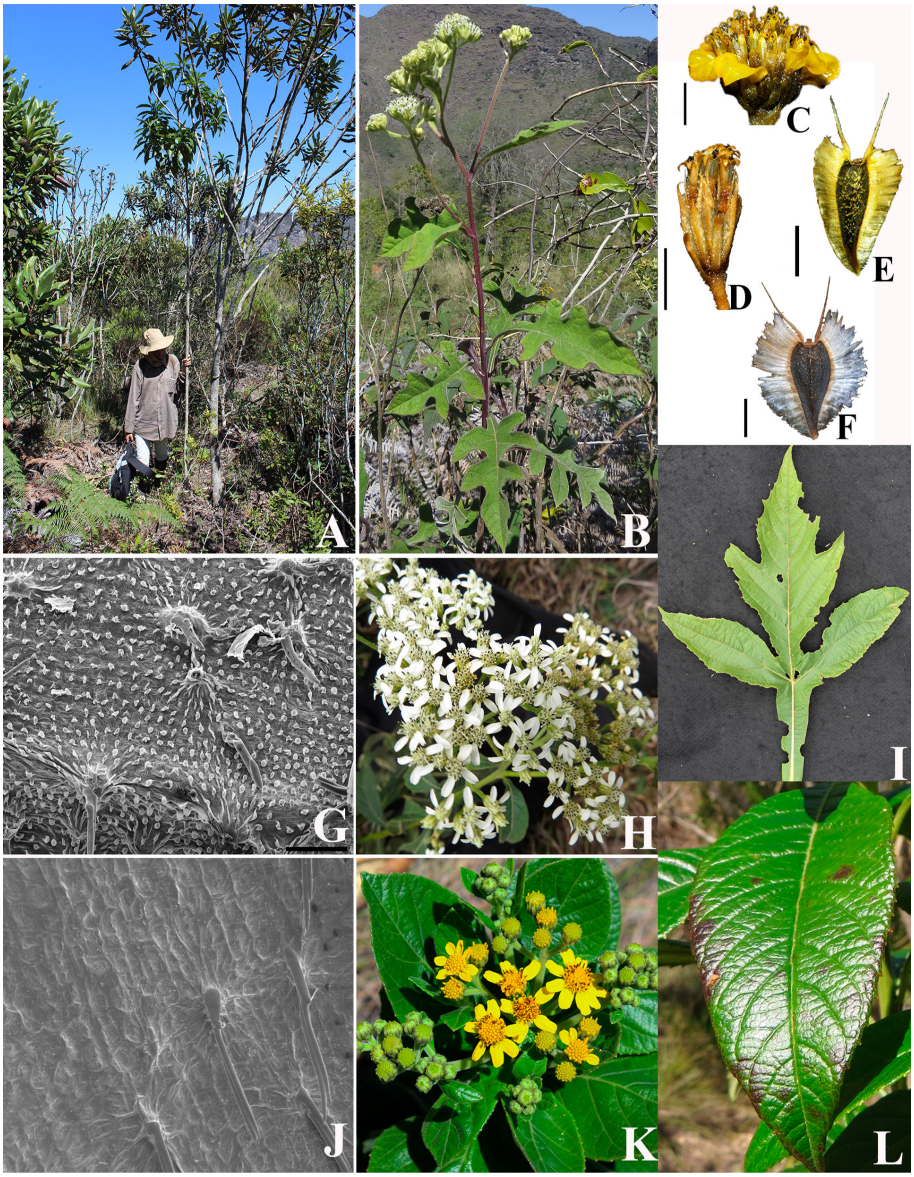

Figure 1. Morphological characters in *Verbesina*. A, Tree of *Verbesina floribunda* Gardner; B, subshrubby habit of *V. bipinnatifida* Baker; C, capitulum of *V. luetzelburgii* Mattf.; D, capitulum of *V. bipinnatifida*; E, cypsela of *V. bipinnatifida*; F, cypsela of *V. floribunda*; G, scanning electron micrograph (SEM) of the verrucous cypsela surface of *V. macrophylla* (Cass.) S.F.Blake; H, inflorescence of *V. macrophylla*; I, pinnatipartite leaf of *V. macrophylla*; J, SEM of the scalariform cypsela surface of *V. glabrata* Hook. & Arn.; K, inflorescence of *V. glabrata*; L, entire leaf of *V. glabrata*. Vouchers: C, *G.L. Moreira* et al. 118 (CEN); D–E, *G.L. Moreira* et al. 116 (CEN); F, *G.L. Moreira* et al. 101 (CEN). Scale bars: C, 8 mm; D, 4 mm; E, 2 mm; F, 4 mm; G and J, 200 µm. Photographs: G. L. Moreira.

restricted to North America. Seven of the 12 sections were subsequently revised (Blake, 1925; Coleman, 1966a, 1966b; Olsen, 1985; Turner, 1985; Olsen, 1988; Turner, 2008).

In the first molecular phylogenetic study of *Verbesina*, based on restriction analysis of chloroplast DNA, 79 species from 11 of the recognised sections (excluding the monotypic *Verbesina* sect. *Stenocarpha* B.L.Rob. & Greenm.) were sampled (Panero & Jansen, 1997). That study indicated that *Verbesina* is monophyletic and is part of a clade comprising mostly Mexican *Podachaenium* Benth., *Squamopappus* R.K.Jansen, N.A.Harriman & Urbatsch, and *Tetrachyron* Schltdl. in the Verbesininae (Panero *et al.*, 1997). Although two large subdivisions (opposite and alternate-leaved clades) were recovered, the sections of *Verbesina* were found not to be monophyletic. Furthermore, a North American origin for the genus was implied, with several distinct introductions into South America, and with the Andes highlighted as a major centre of species diversity (Panero & Jansen, 1997).

Recent family-level molecular studies using chloroplast and nuclear data have included the genus *Verbesina*. One of these studies addressed the pattern of diversification at deep taxonomic levels in Asteraceae, including the tribe Heliantheae, to test the role of dispersal and polyploidy in structuring the existing diversity of the family (Panero & Crozier, 2016). The authors indicated that intercontinental dispersal and polyploidy were important factors in the evolutionary history of the Asteraceae. However, they did not find a close correspondence between genome duplication, dispersal events, and changes in diversification rates in all Asteraceae lineages, and suggested that with denser sampling, increases in the diversification rate for branches that lead to Vernonioid and Heliantheae Alliance clades could be expected to be found, especially in clades that dispersed to South America and radiated extensively (Panero & Crozier, 2016), as is the case for *Verbesina*.

In a phylogenomic study, Zhang *et al.* (2021) analysed changes in diversification rates in the Asteraceae and found six accelerations in rates of diversification in the Heliantheae Alliance. The authors also recorded several genome duplications in species representing all subfamilies and almost all tribes of Asteraceae, including the Heliantheae Alliance. They concluded that independent genome-duplication events affected groups with high species richness, and that such changes probably allowed organisms to take advantage of new ecological opportunities or promoted adaptation to new environmental challenges and therefore may have resulted in their geographical and diversity expansion (Mandel *et al.*, 2019; Zhang *et al.*, 2021).

Here, we present a calibrated molecular phylogeny and biogeographical analysis of *Verbesina* based on DNA sequences of two nuclear markers, and that includes nine Brazilian species and 14 other species not included in the analysis performed by Panero & Jansen (1997), in an attempt to cover the complete geographical distribution range of *Verbesina*. We also aimed to further clarify the infrageneric relationships among *Verbesina* species and thereby improve understanding of the biogeographical patterns associated with the distribution of *Verbesina* in the Neotropics.

Materials and methods

Taxon sampling and distribution data

Sequence data were successfully obtained from 58 different *Verbesina* taxa (57 species and one subspecies). *Podachaenium eminens* (Lag.) Sch.Bip. and *Squamopappus skutchii* (Blake) Jansen, Harriman & Urbatsch were chosen as outgroups, as well as two species of the tribe Heliantheae used in Panero & Crozier (2016), namely *Oblivia mikanioides* (Britton) J.L.Strother and *Otopappus verbesinoides* Benth. (**Supplementary file 2**). The *Verbesina* accessions were chosen from species endemic to North, Central and South America, representing the full geographical range of the genus, and included most of the taxonomic sections (11 of the 12 existing *Verbesina* sections). A distribution map of *Verbesina* species based on records accessed through GBIF (https://www.gbif.org/) was prepared in QGIS 2.12.0 software (**Figure 2**). The elevation images were extracted from the Topodata database (Valeriano & Rossetti, 2012) and the other layers from the IBGE (2019).

DNA extraction, amplification and sequencing

DNA was extracted from fragments, about a thumbnail in size, of leaf tissue either preserved in silica gel or from a herbarium specimen, using a modified CTAB-based protocol (Inglis *et al.*, 2018). DNA quality and integrity were checked using agarose gel electrophoresis, and DNA quantity and purity estimated by NanoDrop UV spectrophotometry (Thermo Scientific, Waltham, MA, USA). The nuclear ribosomal internal transcribed spacer (ITS) and external transcribed spacer (ETS) were selected as markers because they have been shown to be informative in previous studies in other Heliantheae, especially in young lineages (Baldwin & Markos, 1998). Although the authors are aware of the risks of paralogues brought about by incomplete gene conversion with these multicopy markers, no problems with frameshifts or mixed-base polymorphism were observed in the chromatograms of directly sequenced PCR products.

The PCR primers used for amplification of the ETS region were AST-1-mod (5'-CGTAAAGGTGTGTGAGTGGTTT), modified from Markos & Baldwin (2001) to inhibit secondary band amplification, and 18S-Alt (5'-TGAGCCATTCGCAGTTTCACAGTC) (Baldwin & Markos, 1998). For ITS, the primers used were An5 (5'-CCTTATCATTTAGAGGAAGGAG) and An4 (5'-CCGCTTATTGATATGCTTAAA), with the use of the internal primers An2 (5'-GCCGAGATATCCGTTGCCGAG) and U3 (5'-CAWCGATGAAGAACGYAGC), if necessary (Cheng *et al.*, 2016). For ITS, the 15 µL PCR reactions included 1X buffer (GoTaq flexi; Promega, Madison, WI, USA), 2.0 mM $MgCl_2$ and 0.2 mM dNTPs, 1.0 µL of ethylene glycol, 3.0 µL of trehalose (1M), 0.3 µM each of forward and reverse primer, 1 U Taq (GoTaq; Promega) and 1.0 µL of DNA. PCR cycling comprised 2 min at 95°C, followed by 35 cycles of 20 s at 95°C, 40 s at 55°C, and 80 s at 72°C, followed by 7 min at 72°C. The ETS PCR reaction included 2X PCR Buffer (GoTaq flexi; Promega), 2.0 mM $MgCl_2$, 0.2 mM dNTPs,

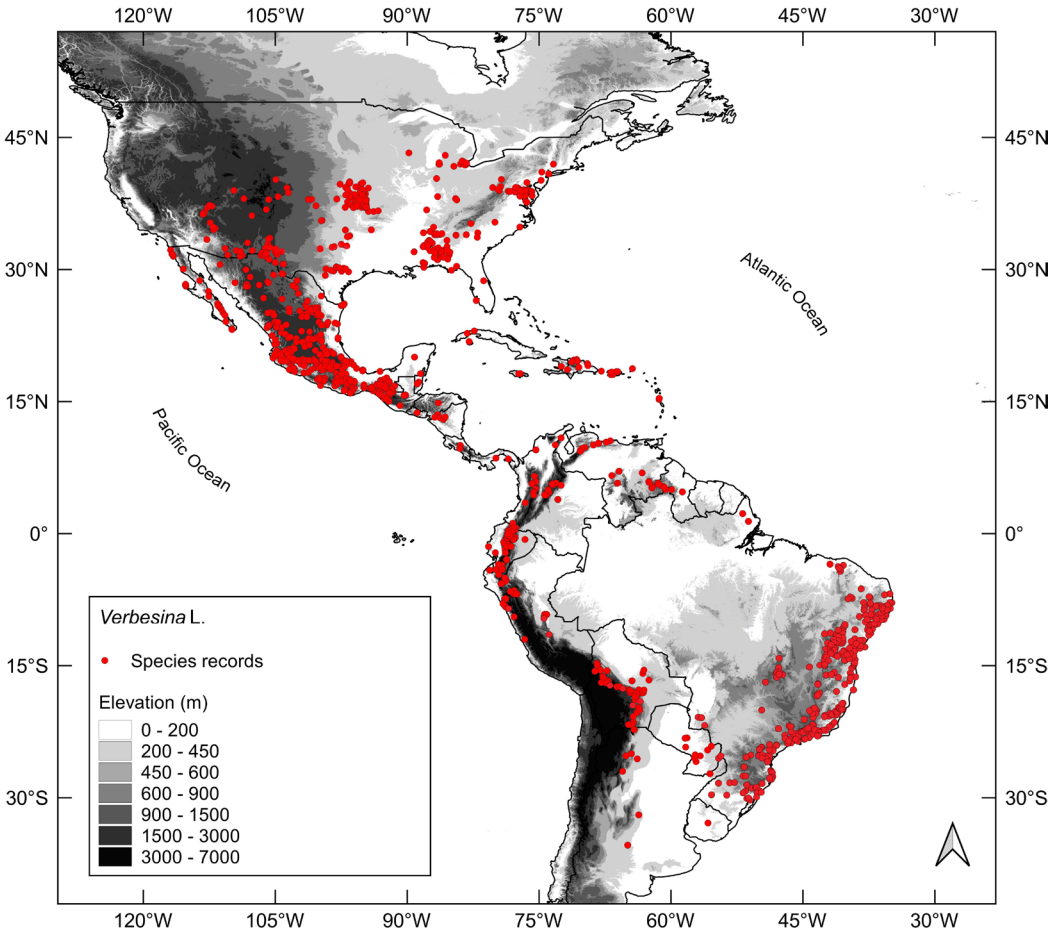

Figure 2. Total distribution of *Verbesina* in North America, Central America, the Caribbean, and South America. (Data accessed from GBIF, https://www.gbif.org/).

0.55 µM each primer, 1 U Taq and 1.0 µL DNA. PCR cycling comprised 1 min at 95°C, followed by 35 cycles of 20 s at 95°C, 30 s at 50°C, and 1 min at 72°C, followed by 7 min at 72°C. PCR products were verified by agarose gel electrophoresis and then prepared for direct sequencing using ExoSAP (ThermoFisher Scientific, Waltham, MA, USA). Both DNA strands were sequenced using the Big Dye version 3.1 kit (Applied Biosystems, Waltham, MA, USA), using the amplification primers as well as internal primers, in the case of ITS.

Phylogenetic analysis

Sequencing products were resolved using an ABI 3730 Genetic Analyzer (Applied Biosystems), and contigs assembled using ChromasPro version 1.5 software (Technelysium, South

Brisbane, QLD, Australia). Sequences were assembled into matrices using BioEdit version 7.2.5 (Hall, 1999) and aligned using the G-INS-i option of Mafft version 7 (Katoh & Standley, 2013). Informative indels were rare in both ITS and ETS matrices, so gap-coding was not applied.

Independent model selections for the ITS and ETS partitions of the combined matrix were accomplished during the run using ModelFinder (Kalyaanamoorthy *et al.*, 2017), part of the IQ-TREE software package (Nguyen *et al.*, 2015). SYM+G4 and TPM3+F+G4 models were found to be optimal according to the Bayesian information criterion for the ITS and ETS partitions, respectively. A phylogenetic hypothesis for the concatenated ITS and ETS matrices was then determined using Bayesian inference (BI) in MrBayes (version 3.2.6; Ronquist *et al.*, 2012). Models for ITS and ETS partitions were unlinked and were optimised over the general time-reversible model space during the runtime using reversible-jump Metropolis-coupled Markov Chain Monte Carlo (MCMCMC) (Huelsenbeck *et al.*, 2004; nst=mixed rates=invgamma). One cold and three heated MCMCMC chains were run for five million generations, sampling every 1000 generations, which was sufficient for the standard deviation of split frequencies to fall below 0.01. Convergence of the analysis was also confirmed using Tracer (version 1.7.1; Rambaut *et al.*, 2018). The first 25% of the trees were discarded as burn-in, prior to calculation of the 50% majority-rule consensus tree.

Divergence dating and biogeographical analysis

A Bayesian time-calibrated phylogenetic hypothesis was constructed using MrBayes 3.2.6 (Ronquist *et al.*, 2012). The dating analysis was calibrated using a log-normal mean age of 12.8 Ma with an offset of 6.03 Ma, according to the date estimated by Panero & Crozier (2016) for the *Helianthus–Montanoa* node. This age is also close to the one obtained by Mandel *et al.* (2019) for the *Verbesina–Montanoa* node of 13 Ma, based on nuclear data. The analysis was performed as in the uncalibrated BI analysis, but additionally using the relaxed independent gamma rate clock under the birth–death speciation process. One cold and three heated MCMCMC chains were run in parallel for five million generations, sampling every 1000 generations, which was sufficient for the standard deviation of split frequencies to fall below 0.01. The first 25% of the trees were discarded as burn-in, prior to calculation of the 50% majority-rule consensus tree.

Our main historical biogeographical question concerned the timing of the development of the large-scale centres of diversity of *Verbesina* in North America and extra-Amazonian South America. We therefore conducted a reconstruction of ancestral area and patterns of dispersal and vicariance, using the Bayesian binary Markov Chain Monte Carlo method (Ali *et al.*, 2012), as implemented in RASP version 3 (Yu *et al.*, 2015), using the post–burn-in trees from the time-calibrated analysis. The calibration used the average dates for the divergence of *Helianthus annuus* L. and *Montanoa revealii* H.Rob. (Heliantheae tribe) (Panero & Crozier, 2016). The defined areas used in the analysis were North and South America, as well as a third connecting area representing Central America and the Caribbean.

Results

Phylogenetic relationships in Verbesina

The BI analysis of combined ITS and ETS sequences strongly supports the monophyly of *Verbesina*, as well as its division into two main clades: A and H (Figure 3). Posterior probability support for the well-resolved tree backbone, as well as many deeper clades (e.g. clades C, I and J), is high. However, several shallower clades (e.g. clades O, K and E) are polytomies or near-polytomies.

Major clade A contains North and western South American (Andean) taxa and comprises two major subclades: clade B, a long branch for *Verbesina encelioides* (Cav.) Benth. & Hook.f. ex A.Gray, distributed in Mexico and the USA; and its sister clade C, in which the species are distributed across the range of the genus, from Mexico to southern extra-tropical South America (Bolivia, Brazil, Ecuador, Peru, Venezuela and Mexico) and the Caribbean. Within clade C, clade D is a polytomy of two predominantly Andean clades and one Mexican clade comprising species with large capitula and leafy phyllaries.

Regarding the two South American Andean clades, clade E (*Verbesina subdiscoidea* Toledo to *V. allophylla* S.F.Blake) includes species with opposite and alternate leaves; and clade G (*V. sodiroi* Hieron. to *V. arborea* Kunth) contains species belonging to *Verbesina* sect. *Lipactinia* B.L.Rob. & Greenm., characterised by small and discoid capitula, alternate or opposite leaves, entire leaf blade, and yellow or white corollas. However, representatives of this section are found in other positions in the phylogenetic reconstruction. Clade F contains representatives of *Verbesina* sect. *Pterophyton* and *Verbesina* sect. *Verbesinaria*, in which *V. corral-diazii* B.L.Turner, *V. pedunculosa* B.L.Rob., *V. curatella* McVaugh, *V. longifolia* A.Gray and *V. pantoptera* S.F.Blake share their herbaceous habit with winged stems, large capitula, and foliose phyllaries.

The second major group in the phylogenetic reconstruction, clade H, repeats the pattern of Mexican basal lineages (clades I, K, M), leading to South American clades (clade N). The sister clades O and Q, comprising taxa from South America, include several representatives of *Verbesina* sections *Lipactinia*, *Verbesina* sect. *Ochractinia* B.L.Rob. & Greenm. and *Verbesina* sect. *Saubinetia* (J.Rémy) B.L.Rob. & Greenm. with small capitula, although representatives of these sections are not exclusively recovered in these clades. However, inflorescences with small capitula occur in all South American clades (see Figure 3). Clade L contains a North American and Caribbean clade M, which is sister to clade N, composed of South American species.

The Brazilian clade O (*Verbesina baccharifolia* Mattf. to *V. floribunda* Gardner) contains species, with alternate, entire leaves and yellow corollas, that are distributed as far as the lowlands and mountain ranges of eastern Brazil, mainly in the Atlantic Rain Forest and Caatinga, although a few are found in the Cerrado. Clade R is an interesting small lineage supported by morphological characters such as deeply pinnatipartite leaves, large

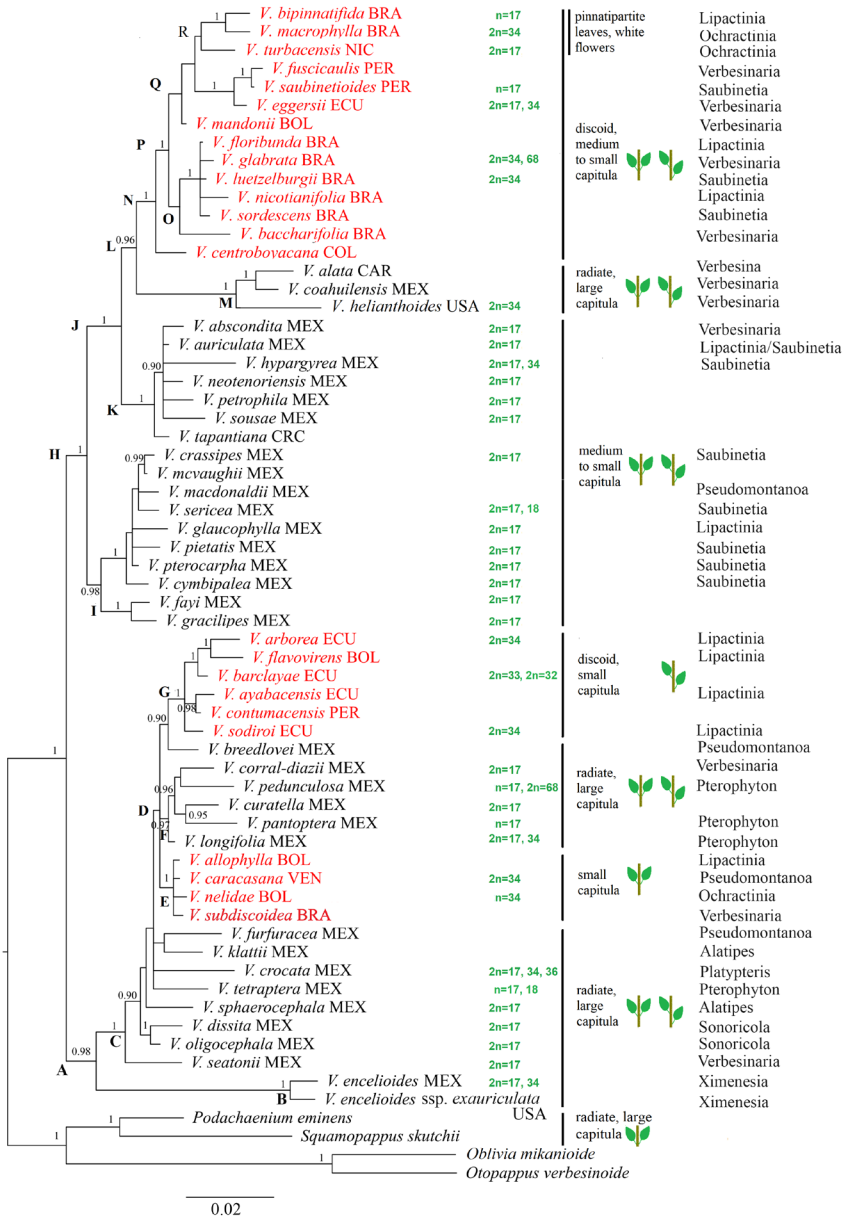

Figure 3. Bayesian inference tree of combined internal and external transcribed spacer data. Posterior probabilities > 0.9 are given above the branches. The red text indicates South American species. Leaf figures indicate opposite and alternate phyllotaxy. Chromosome numbers are based on Moreira & Cavalcanti (2020) and Panero & Strother (2021). BOL, Bolivia; BRA, Brazil; CAR, Caribbean; COL, Colombia; CRC, Costa Rica; ECU, Ecuador; MEX, Mexico; NA, North America; NIC, Nicaragua; PER, Peru; SA, South America; VEN, Venezuela.

inflorescences, white corollas, and cypselas with a unique verrucous surface (see **Figure 1**). White corollas appear only in the South American (Andean and Brazilian) species analysed in this study; this character is found in *Verbesina* sect. *Ochractinia* and *Verbesina* sect. *Lipactinia* and is represented as a homoplastic character in clade E (*V. macrophylla* (Cass.) S.F.Blake var. *nelidae*), clade G (*V. arborea*) and clade R.

Bayesian analysis of ITS and ETS sequences of *Verbesina* yielded a mean date estimate of 8.13 Ma (95% highest posterior density = 5.69–10.53 Ma) for the diversification of *Verbesina* in the late Miocene (Figures **4**, **5**).

Discussion

Monophyly of Verbesina*, morphological variation across clades, and infrageneric classification*

The monophyly of *Verbesina*, as previously demonstrated by Panero & Jansen (1997) using chloroplast restriction patterns, is strongly supported by our BI analysis of combined ITS and ETS DNA sequences. The monophyly of *Verbesina* is also strongly supported in both ITS and ETS gene trees (Supplementary files **3** and **4**, respectively). The division of the genus into two main clades (herein clade A and clade H) was also supported by the results of cpDNA analyses carried out by Panero & Jansen (1997). However, there is no predominance of morphological characters that underpins these two major clades, in contrast to the correlation with opposite or alternate phyllotaxis found in the earlier cpDNA analysis. Only a subtle morphological correlation is observed in clades A and H, in which there is a greater tendency of South American species to have small capitula, as observed in species of clades E, G and N. Clades A and H recovered in our analysis show predominantly North American lineages (mainly from Mexico) leading to South American lineages, and are represented by species from *Verbesina* sect. *Alatipes*, *Verbesina* sect. *Platypteris*, *Verbesina* sect. *Pterophyton*, *Verbesina* sect. *Sonoricola*, *Verbesina* sect. *Verbesinaria* and *Verbesina* sect. *Ximenesia*, all of which share morphological characters such as radiate, medium to large capitula and yellow corollas. Large capitula are more common within *Verbesina*, also present in *Podachaenium* and *Squamopappus* (the outgroups), and somewhat concentrated in North American clades (i.e those containing taxa from Mexico and USA), whereas small capitula and white corollas are concentrated in South American clades.

Prior to molecular studies, hypotheses of infrageneric relationships in *Verbesina* species were based on inflorescence type, capitula size, corolla colour, and presence or absence of ray flowers; these characteristics were used to divide the genus into 12 sections (Robinson & Greenman, 1899; **Supplementary file 1**). Our sequence-based molecular phylogenetic evidence confirms that the current taxonomic sections are not monophyletic, as previously found in the study by Panero & Jansen (1997), and that the characters that define the sections are widely distributed throughout the genus.

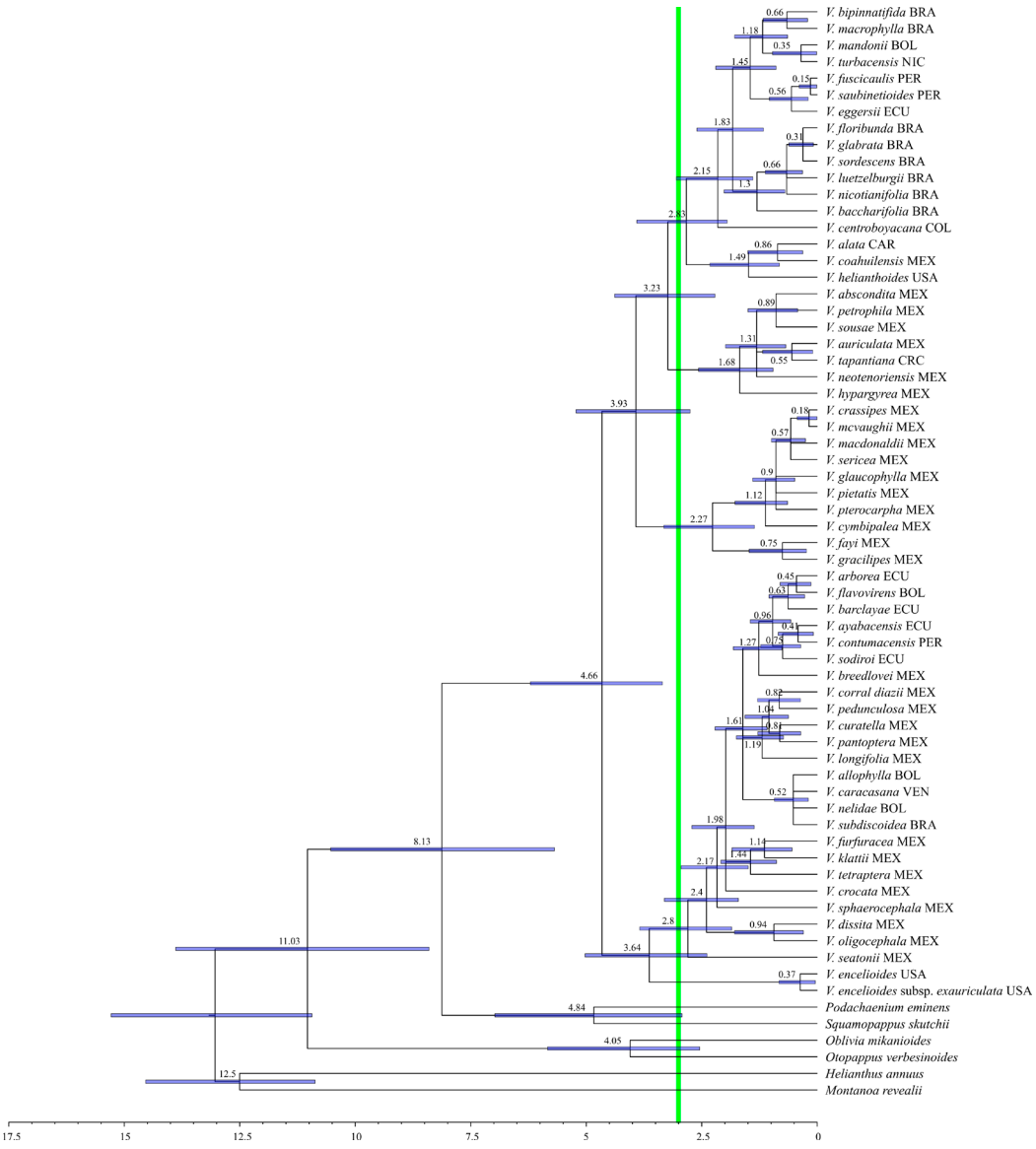

Figure 4. Time-calibrated Bayesian inference tree generated using concatenated internal and external transcribed spacer data. The values at the nodes indicate mean divergence dates, and the horizontal bars indicate 95% highest posterior density ranges for the age at each node. The green bar indicates the consensus age of the closure of the Panama Isthmus (age based on Leigh *et al.*, 2013; O'Dea *et al.*, 2016). BOL, Bolivia; BRA, Brazil; CAR, Caribbean; COL, Colombia; CRC, Costa Rica; ECU, Ecuador; MEX, Mexico; NIC, Nicaragua; PER, Peru; USA, United States of America; VEN, Venezuela.

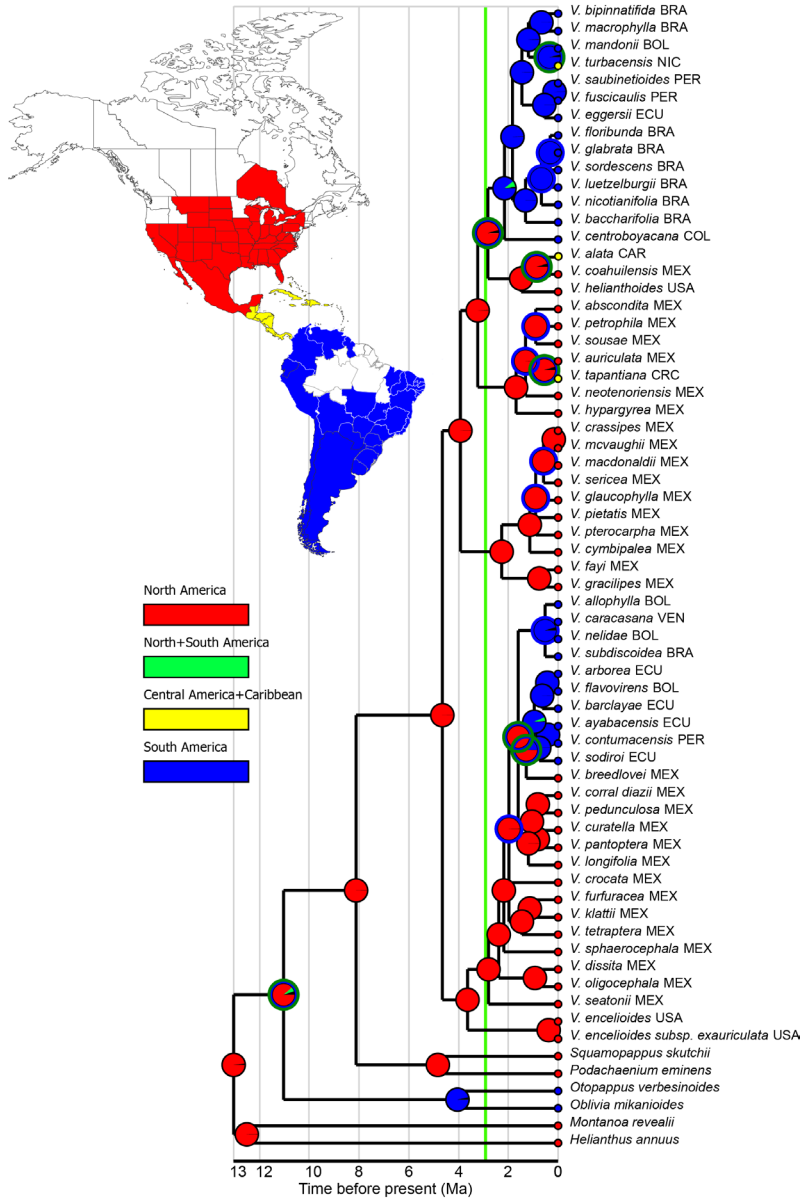

Figure 5. Ancestral area reconstruction produced by means of the RASP–Bayesian binary Markov Chain Monte Carlo method and based on the occurrence of *Verbesina* L. (Heliantheae–Asteraceae) species. Nodes with a predicted dispersal component are circled in blue, and those with a significant vicariance component are circled in green. The green bar indicates the consensus age of the closure of the Panama Isthmus (age based on Leigh *et al.*, 2013; O'Dea *et al.*, 2016). BOL, Bolivia; BRA, Brazil; CAR, Caribbean; COL, Colombia; CRC, Costa Rica; ECU, Ecuador; MEX, Mexico; NIC, Nicaragua; PER, Peru; USA, United States of America; VEN, Venezuela.

The most variable sections morphologically, especially *Verbesina* sect. *Lipactinia*, *Verbesina* sect. *Ochractinia*, *Verbesina* sect. *Pseudomontanoa* B.L.Rob. & Greenm., *Verbesina* sect. *Saubinetia* and *Verbesina* sect. *Verbesinaria* are polyphyletic. However, *Verbesina* sect. *Sonoricola* could represent a monophyletic group and could be redefined in a revised infrageneric classification of the genus. The section is composed of nine species endemic to the Sonoran Desert (Baja California) and northern Mexico, and appears as a monophyletic clade supported by the synapomorphy of long awns in the cypsela.

In clade C, the two representatives of *Verbesina* sect. *Sonoricola* analysed in the present study appear together in a well-supported clade, consistent with the results of the earlier analysis using cpDNA digest patterns (Panero & Jansen, 1997). *Verbesina dissita* A.Gray and *V. oligocephala* I.M.Johnst. share 3–7 mm long awns that are longer than the cypsela body (Coleman, 1966a; Panero & Jansen, 1997), which are apparently an adaptation for dispersal in an arid environment (Panero & Jansen, 1997). Clades E and G comprise species present in the high Andean mountains (of Bolivia, Ecuador, Peru and Venezuela) and the Atlantic Rain Forest in the extreme south of Brazil (*Verbesina subdiscoidea*).

The taxa included in the clade D polytomy, whose four species have white to cream flowers and pinnatifid to pinnatipartite leaves, rarely entire, should be reassessed. The results of morphological analyses by Moreira & Cavalcanti (2020) showed the similarity of *Verbesina subdiscoidea* to the Bolivian species *V. macrophylla* var. *nelidae*. *Verbesina macrophylla* var. *nelidae* is a combination proposed by Olsen (1985), who stated that this taxon is like *Verbesina macrophylla* var. *macrophylla* in the form of its leaf blade, differing in the larger capitula and different types of leaf indumentum. The results presented by Moreira & Cavalcanti (2020) have also indicated that *Verbesina subdiscoidea* may be synonymous with *V. nelidae* Cabrera; however, our molecular evidence does not support this proposal.

Multiple Pleistocene dispersal events into and across South America mark the biogeographical history of Verbesina

A recent time-calibrated molecular phylogenetic study of mostly American species of the Heliantheae alliance (PF clade; Panero & Crozier, 2016) pointed to the diversification of the group from the late Oligocene and early Miocene, around 35 Ma, during times of increasingly drier and colder climate, where today many species of the Eupatorieae, Millerieae and Heliantheae tribes are found in dry areas or mesic montane environments.

A North American origin for the genus is confirmed in our analysis, with at least two distinct dispersals of lineages (clades E + F + G and clade N; see Figures **4**, **5**) into South America, beginning around 3.23 Ma (95% highest posterior density = 1.27–3.23 Ma) in the Middle Pliocene. Diversification in South America began in the Pliocene from 2.83 Ma with the occupation of the Andes. In Brazil, the first introductions seem to have occurred from Andean lineages around 2.15 Ma, exemplified by the deepest branch of the Andean species, *Verbesina centroboyacana* S.Díaz (see Figures **4**, **5**).

The type species of *Verbesina*, *V. alata* L., endemic to the Caribbean, is sister to *V. coahuilensis* A.Gray ex S.Watson, a species from northern Mexico; both share an herbaceous habit, orange to yellow-orange corollas, and cypselae with uncinate awns. The position of these species as sisters helps in understanding the evolution and radiation of *Verbesina* in North America, suggesting that the Caribbean species and those in northern Mexico share a common evolutionary history (Panero & Jansen, 1997).

The deepest-branching species in clade A is *Verbesina encelioides*, which is currently placed in *Verbesina* sect. *Ximenesia* due to its alternate leaves, radiate, large capitula, and yellow to orange corollas. This species is widely distributed in northwestern Andean South America and in much of Mexico and USA in North America (POWO, 2023). The deepest-branching species and subclades of clade C are all endemic to Mexico, which is considered a centre of diversity of the genus (Panero & Jansen, 1997). No South American clade C species penetrates the lowlands of the Brazilian Amazon basin or the Brazilian Cerrado. Dispersal of *Verbesina* species into South America from North America is coincident with biogeographical patterns already reported for other plant groups, such as *Lupinus* L. (Leguminosae) and *Stevia* Cav. (Asteraceae), all of which displaying a historical geographical expansion, possibly facilitated by the closure of the Panama Isthmus (Drummond, 2008; Soejima *et al.*, 2017). This event is widely thought to have facilitated biotic exchange of terrestrial organisms between the Americas (Antonelli & Sanmartín, 2011).

A factor that may have contributed to the rapid diversification in *Verbesina* is uplift of the Andes, which began in the Oligocene, about 25 Ma, and the later of the northern segments, dated to 5–2 Ma (Gregory-Wodzicki, 2000; see also the 2.8–3.0 Ma estimate provided by Leigh *et al.*, 2013 and O'Dea *et al.*, 2016). Studies point to the importance of the Andes as a biological corridor for the dispersal of some lineages of plants adapted to montane conditions, and as a barrier to gene flow between populations in different valleys and low-lying areas (Hoorn *et al.*, 2010) as well as arid regions, such as the Atacama Desert (Antonelli *et al.*, 2009; Luebert & Weigend, 2014). The dates of the closure of the Panama Isthmus (*sensu* Leigh *et al.*, 2013; O'Dea *et al.*, 2016) are coincident with the estimated period of the dispersals of *Verbesina* lineages into South America (see Figures **4**, **5**). The introduction of new lineages led to the rapid expansion and diversification of *Verbesina*, mainly in the Andean region, where the high species richness represents a centre of diversity of the genus alongside Mexico (Panero & Jansen, 1997). Other Asteraceae genera, such as *Stevia* (Eupatorieae), with a geographical distribution pattern similar to that of *Verbesina* in the Americas, have Mexican origins dating to approximately 7–7.3 Ma (Soejima *et al.*, 2017). The dispersal of *Stevia* to Brazil was estimated at 5.2 Ma, which pre-dates recent consensus estimates of the closure of the Isthmus of Panama, in contrast to the post-closure model suggested by our findings for *Verbesina*.

Diversification in *Verbesina* was probably stimulated by dispersal events across humid mesic forest environments, which resulted in occupation of areas of premontane forest

along the base and lower slopes of the Andean mountains that occur from Colombia to the south, with distribution expanding to the Atlantic Rain Forest mountains of eastern Brazil, such as Serra do Mar, Serra da Mantiqueira and Serra do Espinhaço (e.g. *Verbesina bipinnatifida* Baker to *V. macrophylla*, 0.66 Ma).

There is no record of *Verbesina* species for the Brazilian Amazon Forest. The extra-Amazonian distribution of *Verbesina* can be correlated with the distribution pattern of the Andean-centred group of Neotropical plants described by Gentry (1982). These groups are underrepresented in the Brazilian Amazon and in phytogeographical regions of open and dry areas such as Cerrado and Caatinga (*Verbesina baccharifolia*) and in arid environments of Mexico (the *V. dissita* to *V. oligocephala* clade), but they are well represented in the coastal region of Brazil. In Brazil, *Verbesina* species occur predominantly in humid and evergreen forests of the Atlantic Rain Forest biome on the Brazilian coast (*V. bipinnatifida*, *V. glabrata* Hook. & Arn. and *V. macrophylla*) and in gallery forest and high-elevation Cerrado environments (Moreira & Cavalcanti, 2020), which provide habitats for *V. bipinnatifida*, *V. floribunda*, *V. macrophylla*, *V. nicotianifolia* Baker, *V. sordescens* DC. and *V. subdiscoidea*. The occupation of open and dry areas of the Brazilian Cerrado and campos rupestres (open areas of high-elevation mountain tops) is observed for *Verbesina floribunda* and *V. baccharifolia*, respectively (Moreira & Cavalcanti, 2020).

The relationship between the flora of the Andean and sub-Andean areas of Bolivia, Brazilian Pantanal, and Gran Chaco (Argentina and Paraguay) is verified in the phylogenetic reconstruction for *Verbesina*. The occurrence of dispersal events in semideciduous seasonal forests and gallery forests, at 200–1500 m altitude in the Cerrado of Bolivia and Pantanal, indicate a possible dispersal route to the Brazilian Central Cerrado and Atlantic Rain Forest areas of the East Coast of southern Brazil (*Verbesina alophylla* to *V. subdiscoidea*, 0.52 Ma). Further evidence of the expansion of *Verbesina* lineages across the Cerrado to the Atlantic Rain Forest mountains in Brazil is provided by unsupported clade O, containing the strongly supported *V. sordescens* to *V. floribunda* polytomy (0.66 Ma) and *V. baccharifolia*. The expansion of *Verbesina* from the northern Andes (in Colombia, Ecuador and Peru) across the Guiana Shield to eastern Brazil (see Figures 2, 3, 5) is evident throughout clade N (*V. centroboyacana* to *V. bipinnatifida*; 2.15 Ma), a phenomenon reported in other Neotropical plant groups (Gentry, 1982; Cortés & Franco, 1997). This route is consistent with the hypothesis of Pleistocene connections between the disjunct savannas of the Guiana Shield plateaus with the Cerrado in central Brazil, as tested by Werneck *et al.* (2012) for lizard lineages.

By investigating the historical extent of the Cerrado, along with climatic fluctuations in time projections and Quaternary fossil pollen records, the last interglacial (120 ka) models showed evidence of connections in favourable climates between the Cerrado core, Amazonian savannas, and zones of northern transition (Oliveira *et al.*, 2020). A warmer climate would have favoured a more widely distributed Cerrado, including areas in northern

Amazonia and possible eastern coastal connecting routes (Werneck *et al.*, 2012). More recent biotic connections between northern Amazonian savannas and the Cerrado probably occurred along the Atlantic coast, through an Atlantic coast savanna corridor (Silva & Bates, 2002).

Species richness in *Verbesina* may be associated with changes in chromosome number in certain lineages, because polyploidisation is recognised as an important driving force for plant speciation (Alix *et al.*, 2017) and is a common explanation for the successful occupation of new ecological niches (Beest *et al.*, 2012); it facilitates expansion into a wider ecological range compared with that of related diploid species (e.g. Peer *et al.*, 2021). Panero & Crozier (2016) point to polyploidisation occurring in the Oligocene in several Asteraceae lineages, coinciding with an increase in the rate of diversification and appearance of phytomelanic fruits in the clade where the Heliantheae are found, where they associate polyploidisation as well as chromosome losses with radiation of species and niche changes.

For *Verbesina*, there are reports of chromosome counts for 137 species (Strother, 1976; Robinson *et al.*, 1981; Jansen *et al.*, 1984; Carr *et al.*, 1999; Strother & Panero, 2001; Panero, 2007; Moreira & Cavalcanti, 2020; Panero & Strother, 2021). The study by Panero & Strother (2021) indicates that polyploidy in *Verbesina* is evidently more concentrated in the South American lineages, in which polyploidy may be associated with the occupation of new areas, increased ecological tolerance, divergent niche adaptation, and phenotypic novelty (Visger *et al.*, 2016; Karunarathne *et al.*, 2018) and may play an important role in lineage divergence (Machado *et al.*, 2021).

Interestingly, a single 72-bp direct repeat is present at the 5' end of the ETS in all species in clades P, K and I, but not in clade M, causing length polymorphism among ETS amplicons. A similar direct repeat is not present in the included outgroup ETS sequences. The repeats are also imperfect, reinforcing the notion that a single tandem-duplication event occurred in the ETS region, early in the evolution of clade H. In addition to this possible phylogenetic correlation, we did not observe secondary bands among ETS PCR products, nor did we see frameshifts in the directly sequenced chromatograms, suggesting that the observed length variation was not the result of preferential amplification and sequencing of paralogous copies of the marker in the genome. The ETS repeat was possibly lost during the evolution of clade M, either spontaneously or by gene conversion, and possibly following hybridisation with a clade A species. Furthermore, the chromosome count in *Verbesina helianthoides* is 34 (Panero & Strother, 2021), increasing our suspicion that allopolyploidy may have played a role in clade M evolution.

Conclusions

Verbesina is a monophyletic genus, with an extra-Amazonian distribution, that originated in North America and spread to South America following the closure of the Isthmus

of Panama. Dispersal routes through the Andes, and corridors associated with the biogeographical history of the Neotropical savannas, to reach the Atlantic Rain Forest are indicated. The existing infrageneric taxonomic structure of this biodiverse genus with more than 325 species is shown to be unnatural, and we have expanded molecular sampling in South American species to better understand phylogenetic relationships and biogeographical history in *Verbesina* throughout its geographical distribution. The wide Neotropical distribution of *Verbesina*, coupled with its species richness, makes it a great challenge for taxonomic and comparative studies on differences in ecology and morphology between its diploid and polyploid representatives. However, the genus provides an exciting biogeographical model for plant evolution in the region.

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Supplementary material

Supplementary material is available from the *Edinburgh Journal of Botany* online portal.

Supplementary file 1. Infragenic classification of *Verbesina* L. based on Robinson & Greenman (1899), and taxonomic revisions of sections.

Supplementary file 2. Vouchers and GenBank accession numbers of sequences used in phylogenetic analyses. Taxa with name in bold had the sequences generated in the present study; taxa with (*) represent fresh leaves collected in the field.

Supplementary file 3. Maximum likelihood tree of internal transcribed spacer data. Support is indicated by the ultrafast bootstrap values above branches.

Supplementary file 4. Maximum likelihood tree of external transcribed spacer data. Support is indicated by the ultrafast bootstrap values above branches.

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