Phylogenomic Study of *Monechma* Reveals Two Divergent Plant Lineages of Ecological Importance in the African Savanna and Succulent Biomes

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**Abstract:** *Monechma* Hochst. s.l. (Acanthaceae) is a diverse and ecologically important plant group in sub-Saharan Africa, well represented in the fire-prone savanna biome and with a striking radiation into the non-fire-prone succulent biome in the Namib Desert. We used RADseq to reconstruct evolutionary relationships within *Monechma* s.l. and found it to be non-monophyletic and composed of two distinct clades: Group I comprises eight species resolved within the *Harnieria* clade, whilst Group II comprises 35 species related to the Diclipteraeinae clade. Our analyses suggest the common ancestors of both clades of *Monechma* occupied savannas, but both of these radiations (~13 mya crown ages) pre-date the currently accepted origin of the savanna biome in Africa, 5–10 mya. Diversification in the succulent biome of the Namib Desert is dated as beginning only ~1.9 mya. Inflorescence and seed morphology are found to distinguish Groups I and II and related taxa in the Justicioid lineage. *Monechma* Group II is morphologically diverse, with variation in some traits related to ecological diversification including plant habit. The present work enables future research on these important lineages and provides evidence towards understanding the biogeographical history of continental Africa.

**Keywords:** Africa; biome; RADseq; *Monechma*; Justicia; phylogeny; plant diversity

1. Introduction

The Acanthaceae Juss. (Lamiales) are amongst the most diverse and ecologically important vascular plant families in sub-Saharan Africa. They are, for example, the sixth most species-rich family in the Flora of Ethiopia and Eritrea region, the Flora of Tropical East Africa region (Kenya, Tanzania, Uganda), Mozambique and Namibia; the seventh richest in Cameroon and South Sudan; and the ninth richest in Guinea [1–4]. Lineages of Acanthaceae have diversified in a wide range of
habitats ranging from hyper-arid desert to tropical rainforest, and are species-poor only in low-nutrient environments such as on the deep Kalahari Sands of southern Africa and the fynbos of the Cape Floristic Region. In many parts of the continent, Acanthaceae form a dominant constituent of the ground flora such that they provide important ecosystem services and are of economic importance as fodder for livestock and native herbivores [5–7]. Many species of Acanthaceae in sub-Saharan Africa are highly range-restricted and of high conservation concern [6,8–10]. However, despite their obvious importance, our understanding of the diversity and evolutionary history of Acanthaceae is incomplete and many major taxonomic challenges persist [11–16].

One of the most diverse and frequently encountered groups of Acanthaceae in sub-Saharan Africa is the pantropical genus Justicia L., taken in a broad sense (i.e., Justicia s.l.) [17,18]. Although displaying a large range of morphological diversity, plants of Justicia s.l. are readily recognised by the combination of a bilabiate corolla with a rugula (i.e., a stylar furrow on the internal corolla surface), an androecium of two fertile stamens, no staminodes, complex anthers, often with markedly offset thecae and/or with appendages, and 2–4 (–6) colporate pollen with pseudocolpi or with rows of insulae adjacent to the apertures [13,14]. However, recent molecular phylogenetic studies on Justicia and allied genera—together comprising the Justicioid lineage—using evidence from six molecular markers [13,14] have demonstrated that Justicia s.l. is grossly paraphyletic, with several major, morphologically distinct lineages embedded within it. In order to maintain a broadly circumscribed Justicia including morphologically similar taxa such as Anisotes Nees, Anisostachya Nees, Monechma Hochst and Rungia Nees, the entire Justicioid lineage would potentially have to be treated as a single genus [13]. This is highly undesirable as it would require subsuming several species-rich genera that are easily separated morphologically, including Dicliptera Juss. and Hypoestes R. Br. The only plausible alternative, therefore, is to subdivide Justicia s.l. into a number of segregate genera [15]. However, only 12–15% of all members of the Justicioid lineage have been phylogenetically sampled to date and many sampling deficiencies need to be addressed before fully informed taxonomic decisions can be made [13].

One such group highlighted as ripe for further taxonomic work is the genus Monechma Hochst. s.l. (Figure 1) [13]. Monechma, or Justicia sect. Monechma (Hochst.) T. Anderson, is a group of over 40 species confined to continental Africa and Arabia, with the exception of one species (i.e., the type species, M. bracteatum Hochst.) that extends to India (Figure 2). Species of Monechma combine the characters of Justicia listed above with 2- (rarely 4-) seeded capsules bearing compressed seeds with smooth surfaces [19–21]. However, Kiel et al. [13], upon sampling six species (seven accessions) of Monechma, found that this group is not monophyletic and instead separated into two distinct and widely separated clades. Monechma Group I, which includes the type species, falls within the Core Harnieria clade together with members of Justicia sect. Harnieria (Solms-Laub.) Benth. (Figure S1). Monechma Group II, for which only two species were sampled [13], falls within the Diclipterinae clade, sister to core Diclipterinae: Kenyancanthus nudensis (Schweinf.) I. Darbysh and C.A. Kiel + (Hypoestes + Dicliptera) (Figure S1).

Attempts to reconcile this unexpected result with morphological evidence [13] suggested that, based on the limited alternative, the two clades could potentially be separated by differences in inflorescence form. Monechma Group I was considered to be a predominantly tropical African clade in which the flowers are arranged in 1–few-flowered cymes aggregated into axillary and/or terminal spikes or fascicles, with the bracts markedly differentiated from the leaves. Group II, considered to be a predominantly southern African clade, includes species that have single- or rarely 2-flowered (sub) sessile axillary inflorescences, which can together sometimes form weakly defined terminal spikes, but with the bracts largely undifferentiated from the leaves. In a subsequent study of Justicia sect. Monechma in Angola [22], this subdivision was expanded upon and the differences in inflorescence form were used to place the majority of Angolan species within Group I. This included both annual, ruderal species, M. bracteatum and M. monechmoides (S. Moore) Hutch., as well as perennial species of usually fire-prone habitats, such as M. scabridum (S. Moore) C.B. Clarke and allies. That study treated these species within Justicia in view of the uncertainty over application of the name
Monechma but noted that species of Group I may ultimately revert to being referred to under Monechma following more comprehensive molecular studies [22].

Figure 1. Morphological diversity in Monechma s.l. All species pictured have been sampled in the current study. (A) M. monechmoides (E.A. Tripp, Namibia, collected as Tripp and Dexter 787); (B) M. bracteatum (Mozambique, B. Wursten); (C) M. debile (C.A. Kiel, Kenya, Kiel 173); (D) Justicia sp. B of Flora Zambesiaca (B. Wursten, Mozambique, Wursten 1792); (E) M. ciliatum (K. Schumann, Burkina Faso); (F) M. ndellense (A. Thiombiano, Burkina Faso); (G) M. depauperatum (W. McCleland, Mali); (H) M. rigidum (D.J. Goyder, Angola, Goyder 8210); (I) M. virgultorum (D.J. Goyder, Angola, Goyder 8471); (J) M. serotinum (E.A. Tripp, Namibia, Tripp et al. 4068); (K) M. grandiflorum (E.A. Tripp, Namibia Tripp et al. 2034); (L) M. calcaratum (E.A. Tripp, Namibia, Tripp et al. 2043); (M) M. distichotrichum (E.A. Tripp, Namibia, Tripp et al. 2072); (N) M. leucoaderme (E.A. Tripp, Namibia, Tripp et al. 2083); (O) M. mollissimum (E.A. Tripp, Namibia, Tripp et al. 2071); (P) M. desertorum (L. Nanyeni, Namibia); (Q–S) M. divaricatum; (Q) (E.A. Tripp, Namibia, Tripp and Dexter 885); (R) (E.A. Tripp, Namibia, Tripp and Dexter 779); (S) (I. Darbyshire, Namibia); (T) M. genistifolium (I. Darbyshire, Namibia); (U & V) M. cleomoides; (U) (E.A. Tripp, Namibia, Tripp and Dexter 829); (V) (E.A. Tripp, Namibia, Tripp et al. 1960); (W) M. tonsum (E.A. Tripp, Namibia, Tripp and Dexter 813); (X) M. salsola (I. Darbyshire, Namibia, Klaassen et al. 2537). E, F & G reproduced from S. Dressler, M. Schmidt and G. Zizka, African Plants—A Photo Guide: www.africanplants.senckenberg.de [23] with kind permission from the authors and photographers.
Members of *Monechma* s.l. are widespread in sub-Saharan Africa (Figure 2). While a significant number of species of the group occur in fire-prone vegetation corresponding to the savanna biome, the group becomes particularly abundant and diverse (18+ species) in the deserts and shrublands of southwest Africa, centered in southern Angola, Namibia and the Northern Cape region of South Africa [24], which represents one of the main extensions of the succulent biome in Africa [25] (Figure 3). The tropical succulent biome is less well-known than the tropical savanna biome; both experience seasonality in water availability, but the succulent biome differs from savanna in rarely experiencing fire [26]. Within southwest Africa, *Monechma* frequently forms a major component of the dominant ground flora, often in combination with one or more of three other distant related lineages in the Acanthaceae family that have diversified independently in this region: *Barleria* L. (Barlerieae) [7], *Blepharis* Juss. (Acantheae) [5] and *Petalidium* Nees (Ruellieae) [6], with both *Barleria* and *Petalidium* represented by over 25 spp. in Namibia alone [27]. The parallel radiation of species in these four genera within the succulent biome in southwest Africa is remarkable, and together result in the Acanthaceae being amongst the most important plant families in the region. In view of the exceptional ecological importance of these genera, it is essential that we have a strong understanding of the species diversity and evolutionary history of these groups. Taxonomic studies of the Namibian radiation of *Monechma* are ongoing as part of the *Flora of Namibia* programme [28]; however, phylogenetic investigation of the evolutionary history of the group has been lacking to date.
Elsewhere in tropical Africa, members of *Monechma* s.l. can be an important constituent of the fire-prone savanna biome of both the Sudanian and Zambesian phytogeographic regions [29], for example *M. depauperatum* (T. Anderson) C.B. Clarke in the Sudanian region and *M. scabridum* in the Zambesian region. Other species such as *M. bracteatum* and *M. monechmoides* favour open habitats with high light availability and so can be common in disturbed, ruderal environments.

1.2. Aims of the Present Study

The present study intends to reconstruct evolutionary relationships within *Monechma* s.l. in the context of the wider classification of the Justicioid lineage and towards understanding the diversification of this ecologically important lineage. A RADseq phylogenetic approach is used in
light of the considerable success that this method has provided in resolving phylogenetic relationships within other major lineages of Acanthaceae, including Petalidium [6], Louteridium S. Watson [30], Ruelleae [31], Barleria [32] and New World Justicia [33]. The sampling of species of Monechma s.l. is here expanded to include ca. 75% of the accepted taxonomic diversity and, in many cases, to include multiple accessions per species with the goal of assessing reciprocal monophyly of such lineages. Specifically, we aim to (a) test prior delimitation of the two clades of Monechma; (b) identify and/or confirm morphological traits that diagnose the recognised clades; (c) present a first assessment of the biogeographical history of the genus; (d) place all known species of Monechma s.l. into a taxonomic context through a combination of molecular and morphological evidence; and (e) provide a phylogenetic framework to assist with ongoing and future monographic and floristic work on Monechma s.l. and allies in the Justicioid lineage.

2. Materials and Methods

2.1. Sampling

In total, 80 accessions were sampled. Of these, 59 accessions represent 32 of the total 42 species (76%) currently accepted in Monechma or in Justicia sect. Monechma, plus three taxa that are unidentified to species or represent currently undescribed species. The sampling was designed to capture the full range of morphological variation within Monechma s.l. as well as to include two or more accessions of morphologically variable species wherever possible. To help delimit broader-scale relationships, we also included 29 accessions spanning major clades of the Justicioid lineage [13]. Justicia pseudorungia Lindau of the Rungia clade [13] was used as an outgroup for rooting our phylogenetic hypothesis. Leaf tissue for molecular analyses was sampled from either field-collected plant material dried in silica gel or herbarium specimens. Table 1 includes taxon names, source locality and voucher number for all accessions used in this study excluding the removed samples (see section 2.3); these are mapped on Figure 2.

2.2. DNA Isolation and Sequencing Methods

ddRADseq data (double digest restriction-associated DNA) were used to reconstruct phylogenetic relationships among Monechma. At the University of Colorado (Boulder, CO, USA) and Rancho Santa Ana Botanic Garden (RSABG) (Claremont, CA, USA), DNA was extracted from dried leaf tissue using a CTAB protocol [34]. ddRAD libraries were constructed at RSABG using a modified version of that used in [6], which was originally adapted from [35]. A full description of this protocol is published in [6], with details briefly outlined here. All genomic DNA was normalized to ~30 ng/μL before digestion and library construction. Extracted DNA underwent double restriction enzyme digestion using EcoRI and MseI for 3 hours at 37 °C followed by 65 °C for 45 min. Illumina sequencing oligos together with in-line, variable-length barcodes were annealed to the EcoRI cut site and ligated onto digested fragments. Illumina oligos were similarly annealed to the MseI cutsite. Barcoded ligation products were pooled and cleaned using a Qiagen gel extraction kit. We excised fragments from the gel between 200–700 bp to reduce the effects of dimer and to provide more precise amplification of the targeted region. The gel-purified ligations were amplified using the following PCR reaction: 8.6 μL of water, 4 μL of Phusion HF buffer, 0.5 μL of each Illumina primer (10 μM), 0.6 μL DMSO, 0.6 μL DNTPs, 0.2 μL Phusion. Fifteen cycles of PCR were conducted to amplify the cleaned, ligated products. The reaction was repeated once to ameliorate stochastic differences in PCR amplification. Agarose gels were used to assess amplification and size of the PCR products and amplicon concentrations were evaluated using a Qubit fluorometer 2.0. The custom-tagged products of the PCR reactions were pooled and sent to the University of Colorado’s Biofrontiers Next-Gen Sequencing Facility for quality control and further size selection. BluePippin was used to select a fragment range between 200 and 500 bp to reduce the sequenced genome. Libraries from the 80 samples were pooled to yield a final combined library that was submitted for 1 × 75 sequencing on an Illumina NextSeq v2 High Output Sequencer at Biofrontiers.
| Taxon | Source Specimen | Country | Latitude | Longitude |
|-------|-----------------|---------|----------|-----------|
| *Dicliptera maculata* Nees subsp. *usambarica* (Lindau) I. Darbysh. | Kiel et al. 157 (RSA) | Kenya | -0.1791 | 35.6317 |
| *Dicliptera paniculata* (Forsk.) I. Darbysh. | Kiel et al. 166 (RSA) | Kenya | -2.6910 | 38.1639 |
| *Hypoestes forskoahii* (Vahl) R. Br. | Kiel et al. 144 (RSA) | Kenya | -1.8087 | 37.5864 |
| *Hypoestes triflora* (Forsk.) Roem. & Schult. | Kiel et al. 151 (RSA) | Kenya | -0.7033 | 36.4346 |
| *Justicia angulilloides* (Nees) T. Anderson | Kiel et al. 174 (RSA) | Kenya | -3.4144 | 38.4262 |
| *Justicia atenuifolia* Vollesen | Golding et al. 8 (K) | Mozambique | -12.1739 | 37.5494 |
| *Justicia cordata* (Nees) T. Anderson | Kiel et al. 159 (RSA) | Kenya | -2.5514 | 37.8933 |
| *Justicia cubanensis* I. Darbysh. & Goyder | Goyder et al. 8068 (K) | Angola | -14.5897 | 16.9072 |
| *Justicia emnii* Lindau | Bidgood et al. 930 (K) | Tanzania | -7.9167 | 35.6000 |
| *Justicia fanshaweae* Vollesen | Smith et al. 2010 (K) | Zambia | -9.8529 | 28.9441 |
| *Justicia flavia* (Forsk.) Vahl | Kiel et al. 146 (RSA) | Kenya | -1.8082 | 37.5765 |
| *Justicia heterocarpa* T. Anderson | Kiel et al. 158 (RSA) | Kenya | -1.2745 | 36.8146 |
| *Justicia kirkiana* T. Anderson | Kiel et al. 177 (RSA) | Kenya | -3.8407 | 38.6681 |
| *Justicia odorata* (Forsk.) Lam. | Tripp et al. 4073 (COLO) | Namibia | -17.6041 | 12.8872 |
| *Justicia phyllostachys* C.B. Clarke | Bidgood et al. 6871 (K) | Tanzania | -6.7833 | 32.0667 |
| *Justicia platysepalata* (S. Moore) P.G. Mey. | Tripp et al. 4119 | Namibia | -22.3833 | 18.4073 |
| *Justicia platysepalata* (S. Moore) P.G. Mey., (COLO) | Tripp et al. 6907 (COLO) | Angola | -12.8929 | 13.4947 |
| *Justicia platysepalata* (S. Moore) P.G. Mey., Tripp et al. 6919 (COLO) | Angola | -14.9700 | 12.9204 |
| *Justicia pseudorugosa* Lindau | Kiel et al. 185 (RSA) | Kenya | -3.2222 | 40.1218 |
| *Justicia sp. B. of Flora Zambesiaca Bester 11112 (K) | Mozambique | -18.5622 | 34.8731 |
| *Justicea striata* (Klotzsch) Bullock | Kiel et al. 145 (RSA) | Kenya | -1.8082 | 37.5765 |
| *Justicea tetrasperma* Hedrén | Kuharananga et al. 2582 (K) | Tanzania | -6.1994 | 30.3536 |
| *Justicea tricostata* Vollesen | Bidgood et al. 5606 (K) | Tanzania | -8.4500 | 31.4833 |
| *Justicea tricostata* Vollesen | Gillis 11441 (RSA) | Zambia | -15.5470 | 28.2472 |
| *Justicea unguarense* S. Moore | Kiel et al. 163 (RSA) | Kenya | -2.5514 | 37.8933 |
| *Justicea vagabunda* B. Clarke | Tripp et al. 1544 (RSA) | China | 21.9449 | 101.2733 |
| *Kenyaanthus idorensis* (Schweinf.) I. Darbysh. & C.A. Kiel | Lake et al. 17084 (K) | Kenya | -0.1499 | 37.0238 |
| *Monocha australis* P.G. Mey. | Tripp et al. 2028 (RSA) | Namibia | -23.7117 | 17.2600 |
| *Monocha bracteatum* Hochst. | Kiel et al. 161 (RSA) | Kenya | -2.5514 | 37.8933 |
| *Monocha bracteatum* Hochst. | Friis et al. 13345 (K) | Ethiopia | 11.5265 | 35.1075 |
| *Monocha calcaratum* Hochst. | Tripp and Duster 2043 (RSA) | Namibia | -25.8755 | 17.7929 |
| *Monocha ciliatum* Hochst. ex Nees | Merklinger 2013-9-55 (K) | Senegal | 15.3811 | -16.7758 |
| *Monocha cleomoides* C.B. Clarke | Klaassen et al. 2530 (K) | Namibia | -21.2978 | 15.2803 |
| *Monocha cleomoides* C.B. Clarke | Tripp et al. 1995 (RSA) | Namibia | -17.8023 | 12.3261 |
| *Monocha cleomoides* C.B. Clarke | Tripp et al. 1960 (RSA) | Namibia | -19.8212 | 14.1870 |
| *Monocha cleomoides* C.B. Clarke | Tripp et al. 1999 (RSA) | Namibia | -17.5193 | 12.2674 |
| *Monocha debile* Nees | Friis et al. 19459 (K) | Ethiopia | 13.8167 | 39.5500 |
| *Monocha debile* Nees | Kiel et al. 173 (RSA) | Kenya | -3.3496 | 38.4483 |
| *Monocha depauperatum* C.B. Clarke | Etuge 4446r (K) | Cameroon | 5.0833 | 9.7167 |
| *Monocha desertorum* C.B. Clarke | Oliver et al. 6379 (K) | Namibia | -27.4028 | 17.3833 |
| *Monocha distichochilium* P.G. Mey. | Tripp et al. 2067 (RSA) | Namibia | -28.0878 | 19.5131 |
| *Monocha distichochilium* P.G. Mey. | Tripp et al. 2072 (RSA) | Namibia | -27.9074 | 17.6788 |
| *Monocha dioica* C*B. Clarke | Tripp and Duster 808 (RSA) | Namibia | -18.7071 | 17.2921 |
| *Monocha dioica* C*B. Clarke | Tripp and Duster 783 (RSA) | Namibia | -19.5546 | 17.7329 |
| *Monocha dioica* C*B. Clarke | McCade et al. 1275 (RSA) | South Africa | -22.8833 | 29.6667 |
| *Monocha dioica* C*B. Clarke | Tripp et al. 1970 (RSA) | Namibia | -19.6156 | 13.2550 |
| *Monocha dioica* C*B. Clarke | Tripp et al. 2023 (RSA) | Namibia | -23.3475 | 17.0788 |
| *Monocha dioica* C*B. Clarke | Tripp et al. 1961 (RSA) | Namibia | -19.8429 | 14.1279 |
| *Monocha dioica* C*B. Clarke | Tripp et al. 2029 (RSA) | Namibia | -23.7117 | 17.2600 |
| *Monocha dioica* C*B. Clarke | Tripp et al. 2039 (RSA) | Namibia | -26.4395 | 18.1855 |
| *Monocha dioica* C*B. Clarke | Tripp and Duster 4800 | Namibia | -20.3531 | 17.5604 |
| *Monocha genistifolium* C.B. Clarke | Tripp and Duster 775 (RSA) | Namibia | -21.9340 | 16.6867 |
| *Monocha genistifolium* C.B. Clarke | Winnwort & Winnwort 339 | Namibia | -21.5125 | 16.0314 |
| *Monocha grandiflorum* Schinz | Tripp and Duster 2034 (RSA) | Namibia | -24.3024 | 17.8223 |
| *Monocha incanum* C.B. Clarke | Mott 1124 (K) | Botswana | -23.7656 | 22.8097 |
| *Monocha incanum* C.B. Clarke | Puff 780416-2/2 (RSA) | South Africa | -27.9471 | 22.6925 |
2.3. Phylogenetic Reconstruction

We assessed sequencing quality of raw data using FastQC [36]. Data were filtered, trimmed, and demultiplexed using iPYRAD 0.9.31 [37,38]. Of the 80 taxa sampled, four accessions—Monechma sp. (specimen: Tripp et al. 1985), Rhinacanthus angularicus I. Darbysh. (Kiel et al. 170), Justicia flava (Forssk.) Vahl (Kiel et al. 146) and Justicia striolata Mildbr. (Congdon et al. 794)—were removed because of too few loci (i.e., values < 40). Information on the number of ddRAD reads per sample and loci in the assembly for each accession sampled in our study are provided in Table S1. As a result, our final sampling contained 76 accessions, which included 58 accessions of Monechma representing 34 taxa (32 accepted species). Of these taxa, 13 were represented by two or more accessions to account for species with broad geographical distributions and/or variation in morphology (Table 1). The de novo assembly parameters for our final dataset are as follows: the minimum required sequence length (to retain a read) = 35 bp; minimum coverage for retaining a cluster = 6; maximum low quality bases = 5; clustering threshold (level of sequence similarity in which two sequences are identified as homologous) = 0.90; minimum number of samples that must have data at a given locus to be retained = 20; maximum number of alleles per site in consensus sequence = 2. We also conducted 3 additional de novo assemblies exploring the number of minimum samples required to retain a locus (i.e., 4, 10, 30). The final RADseq phylogenomic dataset is available in Sequence Read Archive (SRA) under the BioProject number PRJNA635173.

2.4. Phylogenetic Analyses

We implemented two approaches for estimating phylogenetic relationships among Monechma s.l. 1) a Maximum Likelihood (ML) analysis using the concatenated RAD sequence data from all loci derived from the iPYRAD [37,38] assembly and 2) a coalescent-based approach using quartet-based phylogenetic inference under a multispecies coalescent theory framework that used the concatenated RAD sequence data described above, but randomly sampled one SNP per locus. We conducted our ML analyses using IQ-TREE 1.6.10 [39]. The best model of nucleotide substitution and across-site heterogeneity in evolutionary rates was inferred using ModelTest-NG 0.1.5 [40]. The best-fit model was selected based on the corrected Akaike’s information criterion. Node and branch supports were obtained from 1000 nonparametric bootstrap replicates under the best inferred model (GTR + G). We constructed quartet-based coalescent phylogenetic inferences using the program Tetrad [41] in iPYRAD [37,38] and assessed node support with 1000 bootstraps. The SVDquartets algorithm [42],

| Genus                | Species                  | Locality      | Bootstrap | Support |
|----------------------|--------------------------|---------------|-----------|---------|
| Monechma             | leucoderme C.B. Clarke    | Namibia       | 96         | 0.93    |
| Monechma             | leucoderme C.B. Clarke    | Namibia       | 95         | 0.92    |
| Monechma             | mollissimum (Nees) P.G. Mey. | South Africa | 98         | 0.99    |
| Monechma             | mollissimum (Nees) P.G. Mey. | South Africa | 97         | 0.98    |
| Monechma             | monechmoides (S. Moore) Hutch. | Namibia | 94         | 0.95    |
| Monechma             | monechmoides (S. Moore) Hutch. | Namibia | 93         | 0.94    |
| Monechma             | monechmoides (S. Moore) Hutch. | Namibia | 92         | 0.93    |
| Monechma             | monechmoides (S. Moore) Hutch. | Namibia | 91         | 0.92    |
| Monechma             | rigidum S. Moore         | Angola        | 99         | 0.99    |
| Monechma             | salsola C.B. Clarke      | Namibia       | 98         | 0.98    |
| Monechma             | salsola C.B. Clarke      | Namibia       | 97         | 0.97    |
| Monechma             | salsola C.B. Clarke      | Namibia       | 96         | 0.96    |
| Monechma             | spartioides (T. Anderson) C.B. Clarke | Namibia | 95         | 0.95    |
| Monechma             | spartioides (T. Anderson) C.B. Clarke | Namibia | 94         | 0.94    |
| Monechma             | spartioides (T. Anderson) C.B. Clarke | Namibia | 93         | 0.93    |
| Monechma             | spartioides (T. Anderson) C.B. Clarke | Namibia | 92         | 0.92    |
| Monechma             | spartioides (T. Anderson) C.B. Clarke | Namibia | 91         | 0.91    |
| Monechma             | spartioides (T. Anderson) C.B. Clarke | Namibia | 90         | 0.90    |
| Monechma             | virgulorum S. Moore      | Angola        | 98         | 0.98    |

*Phylogenetic analyses were conducted using iPYRAD and Tetrad.*
implemented in Tetrad [41], uses multi-locus unlinked SNP data to infer the topology among all possible subsets of four samples under a coalescent model. The resulting set of quartet trees are combined and constructed into a species tree. Because the underlying model assumes that the examined SNPs are unlinked, Tetrad subsamples a single SNP from every locus separately for every quartet set in the analysis from the .snps.hdf5 file produced from the iPYRAD output and repeats this subsampling method independently in each bootstrap replicate. This method maximizes the number of unlinked SNP information in the analysis. For both ML and Tetrad analyses, we considered branches to be supported when bootstrap values were >90%, while bootstrap values < 70% were considered unsupported.

2.5. Hypothesis Testing

Six alternative phylogenetic hypotheses were examined using the Shimodaira Approximately Unbiased (AU) tests [43]. Constraint trees were constructed in Mesquite v.2.72 [44]. For each constraint, all aspects of relationships were constructed as a single polytomy, with the exception of the hypothesis under consideration. The constraint trees were loaded into IQ-TREE [39] and run with the settings and model as described above. The best trees from the unconstrained and constrained analyses were combined into a single file and loaded into IQ-TREE and likelihood scores were compared using the AU test with RELL-optimization and 10,000 bootstrap replicates.

2.6. Divergence Time Estimation

To provide temporal context to the evolutionary history of Monechma and close relatives, we estimated divergence times using the most likely tree from our concatenated ML analysis. We pruned this tree to contain a single representative for each ingroup taxon, resulting in a total of 49 species. The singleton tree was rate-smoothed and ultrametricized using penalized likelihood under a relaxed model, where rates are uncorrelated across branches [45] as implemented with the chronos function in ape v 5.1 [46] of R v 3.6.0 (“Planting of a Tree”) [47]. A best-fit smoothing parameter (lambda) of 1.0 was selected following the cross-validation approach and chi-square test as implemented in treePL [48], testing eight values between 0–1000 distributed on a log-scale. A single fossil calibration for a minimum age date of 11.5 my was used to constrain the most recent common ancestor of the Justicioid lineage. This fossil was previously assessed as both reliably identified and dated [49]. Fossil #32 [49] from the Middle Miocene is a dicolporate pollen grain with distinctive round insulae that laterally flank the apertures [50]; the latter of these traits is known only among Justicioids [13,14,51]. We also used a 35 my maximum date for our calibration, which is the estimated age for Justicieae as a whole [49].

2.7. Biome Evolution and Climatic Niche

We reconstructed an ancestral biome state of lineages to elucidate the history of biome occupancy and biome switching in Monechma s.l. For all taxa in our ultrametric tree, species presence/absence in four biomes was determined based on ecoregions [52], as follows: (1) tropical and subtropical grasslands, savannas and shrublands (hereafter savanna biome); (2) deserts and xeric shrublands (hereafter succulent biome); (3) tropical and subtropical broadleaf forests (hereafter forest biome), and (4) montane grasslands and shrublands (hereafter montane biomes). Ancestral state reconstructions were implemented with the rayDISC function in the corHMM 1.13 package [53]. This function assumes a constant rate of evolution across all branches and permits polymorphic character states that account for the probability of either state when calculating the likelihood at ancestral nodes. We compared two distinct Markov models of discrete character evolution: the equal rates (ER) or Mk model, which assumes a single rate of transition among all possible states, and the all rates different (ARD) or the AsyMnMk model [54,55], which allows different rates for each possible transition. We also examined a symmetrical model (SYM), which specifies equal rate transitions in either direction between pairs of states but permits different rates between different pairs. Model fit
was tested by comparing AICc values, from which we selected the model that best fits the data while minimizing the number of parameters [56].

Given asymmetrical patterns of standing diversity in *Monechma* s.l., specifically far greater species richness and abundance in southwestern portions of the range of this lineage, we sought to delimit climatic niche preferences among species throughout the range. We first downloaded 19 WorldClim Bioclimatic variables available in the WorldClim database [57] at 30 arc-seconds resolution [58]. We then extracted bioclimatic data for taxa in our ultrametric tree using latitude and longitude of collections in the R package raster [59]. We visualized changes in two climatic variables: BIO7 = temperature annual range (BIO5 – BIO6: minimum temperature of the warmest month – minimum temperature of the coldest) and BIO12 = annual precipitation (mm), using the *contmap* function in the package *phytools* [60]. The mapping is accomplished by estimating ancestral states at internal nodes using ML with the *fastAnc* function and then interpolating the states along each edge using Equation (2) of [61]; see [62].

2.8. Morphological Studies

A survey of morphological traits that have been found to be taxonomically informative in past studies of both *Monechma* s.l. and the wider Justicioid lineage was conducted for all relevant taxa in order to interpret results of the RADseq analyses. We focused on the following morphological traits: plant habit, inflorescence form, details of the androecium including arrangement of anther thecae and details of the staminal appendages, pollen morphology, and seed number, size, shape and indumentum. Most observations were made on herbarium specimens held at K, RSA and COLO (herbarium abbreviations follow [63]) but with additional observations made via access to digital images of type specimens on JSTOR Global Plants [64] and other online repositories of herbarium specimen images. For pollen morphology, unacetolized pollen from selected taxa was mounted on aluminum stubs using double-sided sticky tape and coated with gold using a PELCO SC-7 system (Ted Pella, Redding, CA, USA). The coated samples were observed at 10 kV on a Hitachi SU3500 (Hitachi, Tokyo, Japan) scanning electron microscope (SEM) at Rancho Santa Ana Botanic Garden. Chromosome number was also considered through reference to relevant cytological studies.

The geographic distribution of each accepted taxon was delimited using the Level 3 codes of the TDWG geographic scheme for recording plant distributions [65].

3. Results

3.1. Phylogenetic Results

The phylogenies inferred using ML for each of the four concatenated data sets were congruent despite variation in the proportion of missing data (Figures 4, S2–S4). The datasets containing more missing data (i.e., larger alignment files with lower min tax values) yielded similar or identical topologies to the datasets containing fewer missing data (i.e., smaller alignment files with higher min tax values; Figures S2 and S3). However, topologies of the latter, in particular the dataset with minimum samples per locus = 30, had lower bootstrap supports for relationships along the backbone of the phylogeny (Figure S4). We here present the results of the concatenated dataset with the minimum samples per locus set at 20 (Figure 4), which contained 5718 loci and 468,892 SNPs. We chose this assembly because it contains the least amount of missing data without losing resolution (see results from Tetrad analysis, below) while also maximizing the amount of genome data utilized.

The coalescent analysis (Figure S5) using the final genotype matrix from the de novo assembly (468,892 SNPs and 20,000,000 quartet sets) resulted in a similar species-level topology to that inferred from the concatenated ML analysis of data. However, the resulting topology inferred from the Tetrad analysis exhibits low resolution along the backbone and thus ambiguous relationships among major clades. Overall, there were no strongly supported topological conflicts between the ML vs. Tetrad analyses (Figures 4 and S5).
Overall, the phylogenetic results from all analyses concur with the findings of the earlier studies [13] (Figure S1) that Monechma s.l. is polyphyletic and that species previously placed in this genus (or in Justicia sect. Monechma) are resolved in one of two clades, with the exception of M. varians (see below). Our data reject strict monophyly of Monechma s.l. (p < 0.001; Table 2).

Monechma Group I (ML: 100% BS; Figure 4) was resolved in the Harnieria clade of the Justicioid lineage and is here composed of six species (M. bracteatum, M. debile, M. monechmoides, Justicia eminii, J. tetrasperma and J. sp. B of Flora Zambesiaca; [18]). This clade is sister to Justicia odora of Justicia sect. Harnieria, and these together are sister to the other four sampled members of sect. Harnieria. Results from an AU test do not reject the monophyly of Justicia sect. Harnieria (i.e., J. unyoensis, J. heterocarpa, J. striata, J. phyllostachys and J. odora; p = 0.393). Species of Monechma Group I for which two or more accessions were sampled (i.e., M. debile, M. bracteatum, M. monechmoides) were each resolved as reciprocally monophyletic (Figure 4).

Monechma Group II (ML: 98% BS; Figure 4) was resolved as a sister to all sampled members of the Diclipterinae clade (i.e., two species each of Dicliptera and Hypoestes; Justicia vagabunda and Kenyacanthus ndorensis). Monechma Group II is here composed of 26 species and includes two major clades. The first clade (ML: 100% BS; Figure 4) consists of six tropical African species (M. ciliatum, M. depauperatum, M. ndellense, M. scabridum, M. subsessile and Justicia attenuifolia). The second major clade (ML: 100% BS; Figure 4) contains the remaining 17 sampled species of tropical and southern African Monechma, primarily those of the succulent biome radiation, in addition to Justicia fanshaweii, J. cubangensis, and J. tricostata, all of which were described in Justicia sect. Monechma. Most species of Monechma Group II for which two or more accessions were sampled (i.e., M. distichotrichum, M. divaricatum, M. genistifolium, M. incanum, M. leucoderme, M. mollissimum and M. salsola) were each resolved as reciprocally monophyletic (Figure 4). Sampled accessions of Monechma cleomoides and M. tonsum were not resolved as reciprocally monophyletic and instead were resolved as part of a clade containing M. genistifolium, M. australis, and M. salsola. Results of an AU test also reject the monophyly of M. cleomoides and M. tonsum (p < 0.001; Table 2).

Monechma varians was here resolved as sister to Justicia kirkiana of the Tyloglossa clade (ML: 96% BS; Figure 4). Together, these taxa are sister to Core Diclipterinae + Monechma Group II and the Harnieria clade, but with weak support (ML: 80% BS; Figure 4).

Justicia platysepala, which was originally placed in Monechma (M. platysepalum S. Moore) but more recently has been included in Justicia [26,66], was here resolved in a clade consisting of J. anagaloides and J. cordata (ML: 94% BS; Figure 4). This clade is sister to all other sampled in-group taxa in our dataset.
Figure 4. The most likely phylogenetic hypothesis for relationships among *Monechma* s.l. generated from ddRADseq loci. *Monechma* s.l. is not monophyletic and is resolved in two major clades: *Monechma* Group I (grey box) and *Monechma* Group II (blue box). The type species, *Monechma bracteatum*, is denoted in Group I. Collection numbers are listed after species names where multiple accessions were sampled. Asterisks [*] indicate 100% ML bootstrap and dashes [-] indicate <70% ML bootstrap.

3.2. Divergence Times

Our divergence time analyses using penalized likelihood estimated that *Monechma* Group I plus *Justicia odora* of the *Harneria* clade originated around 22 mya (stem group) and began to diversify around 18 mya (crown), with *Monechma* Group I specifically diversifying at approximately 12.3 mya (crown; Figure 5). Our analyses estimate that *Monechma* Group II originated around 22.5 mya (stem) and began diversifying around 13.4 mya. Within Group II, however, the succulent biome radiation is estimated to have begun diversifying as recently as 1.9 mya (Figure 5).
Table 2. Results of alternative phylogenetic hypothesis testing using the Shimodaira Approximately Unbiased (AU) test among *Monechma*. H0 = results from present study; H1 = alternative hypotheses based on earlier classification or morphological patterns.

| Hypothesis                                           | logL    | D (logL) | Reject? | p-Value |
|------------------------------------------------------|---------|----------|---------|---------|
| H0. *Monechma* s.l. is not monophyletic              | −1201,860.278 |          |         |         |
| H1. *Monechma* s.l. (excluding *M. varians*) is monophyletic | −1209,690.731 | 7830.5   | Yes     | <0.0001 |
| H0. *Justicia* sect. *Harnieria* is not monophyletic | −1201,860.278 |          |         |         |
| H1. *Justicia* sect. *Harnieria* is monophyletic, i.e., *Monechma* Group I is not embedded within this section | −1201,862.068 | 1.7897   | No      | 0.393   |
| H0. *M. ciliatum* is a member of *Monechma* Group II | −1201,860.278 |          |         |         |
| H1. *M. ciliatum* is not a member of *Monechma* Group II | −1208,510.036 | 6649.8   | Yes     | <0.0001 |
| H0. *M. cleomoides* + *M. tonsum* is not monophyletic | −1201,860.278 |          |         |         |
| H1. *M. cleomoides* including *M. tonsum* is monophyletic | −1203,255.026 | 1394.7   | Yes     | <0.0001 |
| H0. *M. cleomoides* is not monophyletic              | −1201,860.278 |          |         |         |
| H1. *M. cleomoides* is monophyletic                  | −1204,005.563 | 2145.3   | Yes     | <0.0001 |
| H0. *M. tonsum* is not monophyletic                 | −1201,860.278 |          |         |         |
| H1. *M. tonsum* is monophyletic                     | −1203,255.026 | 1394.7   | Yes     | <0.0001 |

3.3. Biome Evolution and Climatic Niche

In our analyses examining transitions among biomes, the common ancestor of the *Harnieria* clade + *Monechma* Group I was most likely distributed in savannas (ER model based on AICc values; Figure 5; Table S2). The ancestor of *Monechma* Group II similarly most likely occupied savannas, with subsequent shifts into the succulent biome including deserts and xeric shrublands (Figure 5). Throughout *Monechma* s.l. and allies, our results suggest there have been rare shifts to tropical forests and montane environments from savanna ancestors (Figure 5).

Ancestral state reconstruction of climatic variables suggests marked shifts in temperature and precipitation regimes during evolution of the succulent biome radiation of Group II. Species in this group (i.e., the “cleomoides” clade) have diversified into drier habitats with greater ranges of temperature extremes in comparison to the others in Group II (Figure 6) as well as the remainder of sampled species in our analyses, including those of *Monechma* Group I.

3.4. Taxonomically Informative Morphological Traits

Our analyses indicated inflorescence form and seed morphology are the most informative morphological characters for separation of *Monechma* Groups I and II. Characters that were found not to be diagnostic for these two clades were morphology of the corolla and androecium and pollen type. Plant habit is not diagnostic but was found to be closely aligned to the phytogeography and ecology of the species within these two clades. Further discussion of results of our morphological survey are presented in the discussion below.

4. Discussion

4.1. Ecology and Biogeography of *Monechma* Groups I and II

Plants of *Monechma* Group I are slender, annual or perennial herbs with brittle leafy stems. In *J. tetrasperma* and sometimes in *J. eminii*, mature plants can be somewhat shrubby with a woody rootstock but they still retain brittle, slender stems. Species of this clade typically favour open habitats with moderate to high light availability, often in areas that regularly burn and can be considered part of the savanna biome. Several of the species, such as *M. bracteatum*, *M. debile* and *M. monechmoides*, favour disturbed, ruderal habitats although they do not become troublesome weeds. Both the range in growth habit and the favoured habitat types observed in *Monechma* Group I is closely similar to that in *Justicia* sect. *Harnieria* to which *Monechma* Group I is closely allied (see section 4.2.2 below).

Plants of *Monechma* Group II vary considerably in growth form (see section 4.2.3), which is again linked closely to ecology. Most species in this clade are perennial herbs or shrublets but *M. ciliatum*, *M. desertorum* and some forms of *M. divaricatum* are annual herbs, though the latter two can be much-
branched. Species of the fire-prone savanna biome in the Sudanian and Zambesian phytogeographic regions [29] are typically perennial herbs that produce fertile shoots from a woody base and rootstock that are burnt back during the dry season (similar to geoxylic suffrutices). The exception is *M. ciliatum*, which does not perennate. Species from drier, non-fire prone habitats, particularly in the deserts and xeric shrublands comprising a major extension of the succulent biome in southwest Africa, are most often shrublets with intricate branching (Figure 3).

![Figure 5](image)

*Figure 5.* Divergence time estimation from penalized likelihood and biome evolution for *Monechma* Groups I and II. Ancestral state reconstruction: circles at nodes are color coded to reflect ancestral character states with sizes of differently colored wedges indicating likelihood of presence of each state at that node.

The majority of species in *Monechma* s.l. occur in the savanna biome and this is reconstructed as the ancestral biome state of the lineage (Figure 5). Our analyses suggest the origin of the lineage at ~31 mya, but the savanna biome is thought to have originated 5–10 mya with the spread and increased dominance of fire-prone C4 grass lineages [67,68]. Indeed, in Africa, phylogenetic evidence suggests that the origin of most lineages of ‘underground trees’ (geoxylic suffrutices) that place their woody biomass underground to protect it from fire dates to within the last 2 myrs, after the origin and spread of the savanna biome [69]. Either previous studies have grossly inaccurately dated the timing of the origin of the fire-prone savanna biome or *Monechma* s.l. originated in some other biome, with most lineages subsequently shifting to the savanna biome once that biome as we now know it originated; c.f. [70] (figure 5b). Under the latter scenario, the previous biome(s) occupied by *Monechma* s.l. may have no modern-day analogue, while species in the lineage may have possessed traits that predisposed them to successfully colonise the savanna biome; c.f. [71].
While figuring out the exact timing of colonisation of the savanna biome by *Monechma* s.l. may require further paleobotanical and geological evidence, it seems likely based on our analyses that the ancestors of most extant species were found in savanna except for the conspicuous radiation of >15 species in *Monechma* Group II within the succulent biome (Figure 5). The latter clade, predominantly composed of species in Namibia and neighbouring countries, may have originated as early as ~7 mya (stem age), but seems to have begun substantial diversification within the last one million years (crown age for clade comprising 14 of the 15 species phylogenetically sampled in the clade) (Figure 5). The recency of this radiation is reminiscent of radiation of a distantly related genus of Acanthaceae, *Petalidium*, which similarly has undergone very recent radiation in the succulent biome in southwest Africa, with 39 species originating in the last 0.5 myrs [6]. Indeed, it has been argued that arid environments can facilitate rapid diversification of plant lineages [72], including a suggestion that such has been the case for *Monechma* in this region [24]. These results are surprising, however, as the Namib Desert, which forms the core of the distribution of the succulent biome in southwest Africa, is thought to be among the oldest deserts on Earth, dating to at least 55–80 mya [73]. Clearly, further research, phylogenetic and otherwise, is needed to understand the biogeographical history of this understudied, yet biologically unique region.

4.2. Taxonomic Implications

4.2.1. Morphological and Cytological Traits for the Separation of *Monechma* Groups I and II

Our results confirm that *Monechma* s.l. is a non-monophyletic assemblage of species from two widely separated clades within the Justicioid lineage. In line with earlier studies [13], *Monechma* Group I is nested within the Core *Harniera* clade whilst *Monechma* Group II is allied to the Diclipterinae clade (Figure 4). However, the constituent species of the two clades of *Monechma*, as revealed by our detailed sampling, do not concur with that interpreted from the limited sampling in earlier studies [13,22]. Specifically, the majority of newly sampled species from tropical Africa with primarily terminal inflorescence spikes bearing the bracts ± highly differentiated from the leaves were not resolved among the *Monechma* Group I clade (Figure 4), as was inferred by those earlier studies [13,22]. Instead, they are resolved in a series of clades within *Monechma* Group II, which is otherwise
made up of predominantly southern African species with ± undifferentiated bracts. Thus, the enumeration of *Monechma* Group I in Angola [22] includes taxa from two clades. In light of the present results, the morphological grounds for the separation of the *Monechma* Group I and Group II requires reassessment.

Although quite variable across *Monechma* s.l., growth habit is not diagnostic for separation of these two clades, instead being more closely linked to ecology, which varies particularly within Group II (see Section 4.1) (Figures 5 and 6). Similarly, we do not find traits in the corolla and androecium to be informative. In fact, these traits are remarkably uniform across *Monechma* Group I and II and also across allied taxa in the wider Justicioid lineage, for example in the *Harnieria* and *Tyloglossa* clades. All have the combination of a short and relatively broad corolla tube with tube length usually ≤ the lips (rarely tube > lips e.g., in *M. grandiflorum*) and with prominent transverse ridges (“herring-bone” patterning) on the lower lip (Figure 1); and anthers with offset and often oblique thecae with a prominent pale appendage on the lower theca. In both *Monechma* Groups I and II, the appendage is often bifurcate or even trifurcate at the apex, but this is inconsistent and anthers on the same plant can have entire and divided appendages.

Pollen morphology is often informative in the classification of Acanthaceae [13,14,74], hence pollen morphology has previously been reviewed across the Justicioid lineage [13]. That study found that members of the *Harnieria* clade, including *Monechma* Group I, have bicolporate pollen with each aperture flanked by lines of insulae; see [13] (figure 11C, D, G and H). The same pollen type was found in the only sampled member of *Monechma* Group II, *M. divaricatum*. We examined one additional species from *Monechma* Group I and two additional species from Group II and further confirm these results (Figure 7). Therefore, pollen type does not distinguish between the two Groups. However, it is noteworthy that this pollen type in Group II is different from other sampled members of Diclipterae in which the pollen is usually tricolporate-hexapseudocolpate, although in *Rhinacanthus virens* (Nees) Milne-Redh. the pollen is tricolporate with insulae; see [13] (figure 11).

Inflorescence morphology varies considerably within the Justicioid lineage and has been used as an important character in past classification schemes, e.g., [51]. There is considerable variation in inflorescence form in *Monechma* s.l. [19], but based on results presented herein, separation of the two clades using this character is not as straightforward as that proposed in earlier studies [13,22]. In *Monechma* Group I, the flowers are held in axillary or mixed axillary and terminal spikes in which the bracts are highly differentiated from the leaves and are ± broadly elliptic, ovate or obovate (Figure 1A–D). Only in rare cases in Group I is the terminal spike longer than the axillary spikes (or more rarely axillary spikes are absent, e.g., in some specimens of *M. debile*). Each inflorescence unit within the spike can have a single flower, but often contains two or more flowers. The inflorescence arrangement of *J. tetrasperma* has previously been analyzed in detail [19]. That study found that the dichasial units in the proximal portion of the spike have two pairs of bracts, the upper pair of which are highly uneven in size (i.e., three conspicuous bracts and one inconspicuous bract), while units in the distal portion of the spike have only one ± equal pair of bracts. This arrangement is not observed in *Monechma* Group II where the inflorescences are simpler: most species have single-flowered (rarely 2-flowered) inflorescence units per bract, with either one or two inflorescence units per node. In most species from the succulent biome of southern Africa (e.g., *M. cleomoides, M. divaricatum* etc.), the bracts are ± undifferentiated from the leaves and so the flowers are axillary (Figure 1K–S, U–X), although, in some species (e.g., *M. genistifolium*, Figure 1T), they form weakly defined terminal spikes. Most species from savanna biome in tropical Africa have bracts that are clearly differentiated from the leaves and have flowers that are held in well-defined terminal spikes, occasionally with additional, usually shorter spikes in the distal-most leaf axils (Figure 1E–I). The only known exception to this is *J. fahnslawei*, which has short axillary and terminal spikes (primarily the former). Species of Group II with well-defined spikes typically have bracts that are proportionately narrow and linear or lanceolate. *Justicia kasamae* Vollesen from Zambia (not sampled in our RADseq analysis but included in Group II on the basis of morphology) is an exception, having imbricate bracts that are broad and elliptic to obovate [18].
Capsules of both clades of *Monechma* typically have only two seeds developed due to early abortion of the upper two ovules [19], although all four ovules mature in *J. tetrasperma* of *Monechma* Group I. Seeds are uniform in *Monechma* Group I, being small, 2–3 mm in diameter [17,18], lenticular with a sharp rim and mottled surface, ± symmetrical in cross section and lacking a ridge on one surface, and are glabrous (Figure 8A–C). Seeds of *Monechma* Group II are much more varied in terms of size, shape, surface characteristics and indumentum (Figure 8D–J); this is discussed in more detail in Section 4.2.3. In summary, the seeds of many species in Group II are larger than in Group I and/or they are less strongly compressed with a more rounded rim; they are often asymmetric in a cross section and often have a ± conspicuous longitudinal ridge on one side. Seed colour varies from black to mottled grey or sometimes (e.g., in *M. divaricatum*; Figure 8I) intricately patterned and coloured. Seeds can be glabrous or can have trichomes (Figure 8D,E). Critically, those species of Group II with small, lenticular, glabrous seeds (e.g., *M. desertorum*, Figure 8H) are ± markedly asymmetric in a cross section, with one surface convex and the other often flat or even slightly concave, and have a conspicuous ridge on one side, quite unlike those of Group I.

Chromosome number has also been found to vary considerably across Acanthaceae [75–78]. However, as far as is known, very few chromosome counts are available for *Monechma* s.l. Within *Monechma* Group I, two independent counts of 2n = 28 have been reported for *Justicia debilis* Lam. (= *M. debile*) [79]. Counts of 2n = 26 and 2n = 28 are common in *J.* sect. *Harniera* to which *Monechma* Group I is closely allied (and also other Old World *Justicia*), but the count within that clade is variable and some species have 2n = 40 (~50) [76]. Within *Monechma* Group II, a count of n = 11 was recorded for *M. ciliatum* [75]. This differs notably from the count of n = 15, which is otherwise characteristic of the Diclipterinae clade [13]. Further studies are required to confirm the consistency of the chromosome counts within, and differences between, the two *Monechma* clades.
In summary, the morphological and cytological differences between the two widely separated clades of *Monechma* are subtle and diagnosis is somewhat hindered by significant morphological variation within each clade, particularly among Group II. However, differences in inflorescence form and seed characteristics, potentially together with differences in chromosome number, are informative in separating these two clades. The constituent species for these two clades are listed in Table 3, with those species not sampled in the RADseq phylogeny being placed based on their morphology.

4.2.2. Relationship of *Monechma* Group I to *Justicia* sect. *Harnieria*

Our results confirm a close relationship between *Monechma* Group I and *Justicia* sect. *Harnieria* (henceforth sect. *Harnieria*). Sect. *Harnieria* is found to be paraphyletic, with *J. odora* being sister to *Monechma* Group I, although monophyletic of sect. *Harnieria* cannot be rejected ($p = 0.393$; Table 2). This result concurs closely with the findings of earlier studies [13], where a larger sample of species of sect. *Harnieria* was included than in the current study, and where *J. capensis* Thunb. and *J. odora* together were found to be sister to *Monechma* Group I. A number of morphological similarities have been noted between sect. *Harnieria* and *Monechma* s.l. [19], including general corolla shape, presence of conspicuous transverse ridges (“herring-bone” patterning) on a large portion of the lower corolla lip, and biaperturate pollen with insulae, as well as a similar inflorescence form between some members of *Monechma* and sect. *Harnieria*. These similarities, together with the fact that *J. tetrasperma* has an intermediate fruit type, have been used in support of reducing *Monechma* s.l. to a section of *Justicia* [19].
Table 3. Currently accepted species in *Monechma* s.l., their placement in *Monechma* Groups I and II and their distribution. ^ denotes that the species has been sampled in the current RADseq phylogeny. Species that were not sampled have been placed based on morphology; placement of these taxa in the clades of *Monechma* Group II should be considered provisional. Combinations in *Monechma* are used whenever available, in preference to combinations in *Justicia*. Geographic range follows TDWG Level 3 codes [65].

| Clade                        | Constituent Species                                             | Distribution                                      |
|------------------------------|-----------------------------------------------------------------|---------------------------------------------------|
| *Monechma* Group I           | ^*Monechma bracteatum* Hochst.                                    | Africa: ANG, BOT, ERI, ETH, KEN, MLW, MOZ, NAM, NAT, SOM, SUD, TAN, TVL, UGA, ZAI, ZAM, ZIM; Asia: IND, OMA, YEM |
|                              | ^*Monechma dehile* (Forssk.) Nees                                 | Africa: DJI, ERI, ETH, KEN, SOM, SUD, TAN; Asia: SAU, YEM |
|                              | ^*Monechma monechmoides* (S. Moore) Hutch.                       | Africa: ANG, BOT, MLW, MOZ, NAM, TVL, ZAM, ZIM    |
|                              | Justicia carnosa Hedrén                                           | Africa: BUR, MLW, RWA, TAN, UGA, ZAI, ZAM         |
|                              | *Justicia eminii* Lindau                                          | Africa: MOZ                                         |
|                              | ^*Justicia tetrasperma* Hedrén                                    | Africa: TAN, ZAI, ZAM                             |
|                              | *Justicia sp. B of Flora Zambesiaca                               | Africa: MOZ                                         |
|                              | *Justicia sp. C of Flora Zambesiaca                               | Africa: ZAM                                         |
| *Monechma* Group II           | ^*Monechma ciliata* (Jacq.) Milne-Redh.                          | Africa: ANG, ZAI, ZAM                             |
| “ciliatum/scabridum” clade    | ^*Monechma virgularum* S. Moore                                   | Africa: ANG                                         |
|                              | ^*Justicia atenuifolia* Vollesen                                  | Africa: MOZ                                         |
| “virgularum” clade            | ^*Monechma glaucifolium* S. Moore                                | Africa: ZAM                                         |
|                              | *Monechma lolioides* (S. Moore) C.B. Clarke                      | Africa: ANG                                         |
|                              | ^*Monechma rigidum* S. Moore                                     | Africa: ANG                                         |
|                              | ^*Justicia cubangensis* I. Darbysh. & Goyder                      | Africa: ANG                                         |
|                              | Justicia eriniae I. Darbysh.                                     | Africa: ANG                                         |
|                              | Justice laet S. Moore                                            | Africa: ANG                                         |
|                              | ^*Justicia triostata* Vollesen                                    | Africa: TAN, ZAM                                   |
| “serotinum” clade             | ^*Monechma serotinum* P.G. Mey.                                  | Africa: NAM                                         |
| “cleomoides” clade            | ^*Monechma australis* P.G. Mey.                                  | Africa: CPP, NAM                                   |
|                              | ^*Monechma calcaratum* Schinz                                     | Africa: NAM                                         |
|                              | *Monechma callohamnum* Munday                                     | Africa: NAM                                         |
|                              | *Monechma cleomoides* (S. Moore) C.B. Clarke                     | Africa: ANG, NAM                                   |
|                              | Monoechma crassiusculum P.G. Mey.                                | Africa: NAM                                         |
|                              | ^*Monechma desertorum* (Engl.) C.B. Clarke                      | Africa: NAM                                         |
|                              | ^*Monechma disaricatum* (Nees) C.B. Clarke                      | Africa: ANG, BOT, CPP, CPV, MOZ, NAM, NAT, OFS, SWZ, TVL, ZAM, ZIM |
|                              | ^*Monechma distichotrichum* (Lindau) P.G. Mey.                   | Africa: CPP, NAM                                   |
|                              | ^*Monechma genistifolium* (Engl.) C.B. Clarke                    | Africa: NAM                                         |
|                              | ^*Monechma grandiflorum* Schinz                                   | Africa: NAM                                         |
|                              | ^*Monechma incanum* (Nees) C.B. Clarke                           | Africa: BOT, CPP, NAM, OFS                         |
|                              | *Monechma leucoerme* (Schinz) C.B. Clarke                        | Africa: NAM                                         |
|                              | ^*Monechma mollissimum* (Nees) P.G. Mey.                         | Africa: CPP, NAM                                   |
|                              | Monoechma robustum Bond                                          | Africa: CP                                          |
|                              | *Monechma salzola* (S. Moore) C.B. Clarke                       | Africa: ANG, NAM                                   |
|                              | Monoechma sattalile Munday                                       | Africa: CPP                                         |
|                              | ^*Monechma spartioides* (T. Anderson) C.B. Clarke                | Africa: CPP                                         |
|                              | ^*Monechma tossum* P.G. Mey.                                     | Africa: NAM                                         |
| *Monechma* Group II incertae sedis | Justicia kasamae Vollesen                                    | Africa: ZAM                                         |
The principle difference between *Monechma* Group I and sect. *Harnieria* is in the fruits. *Monechma* Group I usually have 2-seeded capsules (4-seeded in *J. tetrasperma*) and seeds with a smooth testa. Those of sect. *Harnieria* have 4-seeded capsules with tuberculate seeds, although some species are heterocarpic with highly modified single-seeded indehiscent fruits in addition to the typical dehiscent capsules [13,76].

Variation in sculpturing of the seed testa has been observed within other lineages of Acanthaceae. For example, apparently closely allied members of the genus *Isoglossa* Oerst. in East Africa can have either a rugose testa (e.g., *I. floribunda* C.B. Clarke, *I. grandiflora* C.B. Clarke) or smooth testa (e.g., *I. mbalensis* Brummitt, *I. uifensis* Brummitt) [80]. Furthermore, within the Justicioid lineage, seeds with a smooth testa are not unique to the two clades of *Monechma*: smooth seeds are observed in several other taxa in *Justicia* s.l. apparently unrelated to the two clades of *Monechma*. These include the group of species *J. grisea* C.B. Clarke, *J. rendleii* C.B. Clarke and *J. salvioides* Milne-Redh. from East Africa, and *J. crebrinodis* Benoist and allies from Madagascar. The *J. crebrinodis* group also have 2-seeded capsules, but are otherwise very different morphologically to the clades of *Monechma* and molecular phylogenetic evidence confirms that they are not closely related [81]. This evidence suggests that variation in seed number and sculpturing may hold only limited taxonomic value at the generic rank within the Justicioid lineage and that it might, therefore, be advisable to treat *Monechma* Group I and sect. *Harnieria* as a single taxonomic unit. Nevertheless, further studies, including more thorough molecular sampling of sect. *Harnieria*, are required to fully decipher relationships within that group and in relation to *Monechma* Group I.

4.2.3. Morphological Variation within *Monechma* Group II

As noted in Section 4.2.1, *Monechma* Group II as recircumscribed here includes a range of morphological variation. Based on the results of the RADseq phylogeny, two major clades are noted (see Results), the latter of which contains several minor clades that can be delimited on morphological grounds and may form the basis for a future classification; these are summarised below. The constituent species for each of these clades are listed in Table 3, with those species not sampled in the RADseq phylogeny being placed based on their morphology.

(i) The “ciliatum/scabridum” clade, which contains species of fire-prone savanna biome in tropical Africa, largely associated with the Sudanian and Zambesian phytogeographic regions [29]. These species all share terminal spiciform inflorescences (sometimes with additional spikes in the uppermost leaf axils) and bracts that are ± highly modified from the leaves in both size and shape. The widespread West and Central African species *Monechma ciliatum* is unique in this clade in being an annual herb and in having unusual bristly trichomes on the seeds, restricted to tufts at the apex and base of the seeds, the two tufts being oriented in opposite directions (Figure 8E). All other species in this clade, such as *M. depauperatum*, *M. scabridum* and *M. subsessile*, are suffruticose herbs (see Section 4.1). Their seeds are at first finely white-puberulous but later glabrescent. They are rounded or oblate in face view and are compressed but with rounded margins and have one face concave when young, the other face convex and with a ± conspicuous central ridge (Figure 8D). Our data reject the exclusion of *M. ciliatum* from *Monechma* Group II (*p* < 0.001; Table 2).

(ii) The “virgulorum” and (iii) the “tricostatum” clades, which comprise suffruticose herbs of southern tropical Africa, mainly associated with the Zambesian phytogeographic region [29]. As in the “ciliatum/scabridum” clade, they have predominantly terminal spiciform inflorescences with highly modified bracts, the exception being *Justicia fanshaweii*, which has short axillary and terminal spikes. The seeds in these clades are less compressed than in the “ciliatum/scabridum” clade and are glabrous (Figure 8F,G). Several members of these two clades have secund inflorescence spikes in which only one of each pair of bracts is fertile, but this is not universal, for example both *M. rigidum* and *J. tricostata* can have opposite flowers along the spike. The “tricostatum” clade differs from the “virgulorum” clade in having prominently 3-veined calyx lobes, bracts and bracteoles, the veins often being a markedly different colour from the intercostal surfaces. In *M. virgulorum* and *M. fanshaweii*, the calyces are at most only weakly 3-veined with only the midvein ever prominent.
(iv) The “serotonin” clade. *Monechma serotoninum*, a rare species endemic to the Kaokoveld of Namibia, occupies a position in the phylogeny between the tropical African, fire-prone savanna clades outlined above and the group of species that are concentrated in the non-fire prone deserts and bushlands of southern Africa, i.e., the “cleomoides” clade discussed below. *Monechma serotoninum* is also somewhat intermediate in morphological terms. It has a well-defined lax terminal spike with reduced bracts in comparison to the leaves as in most members of the savanna clades, but it has the dwarf shrubby habit of many species of the “cleomoides” clades (see below), in keeping with its non-fire prone habitat. The seeds of this species are glabrous, compressed, and asymmetric in cross section, with one face convex.

(v) The “cleomoides” clade. The remainder of the taxa in *Monechma* Group II are included in a single, large clade which comprises southern African taxa of dry, non-fire prone habitats including deserts and bushlands of the succulent biome. Most of the species are dwarf shrublets, often intricately branched and sometimes with gnarled lignified mature branches, although *M. desertorum* and some forms of *M. divaricatum* are annual herbs [20]. These species are united by having single-flowered axillary inflorescences with the bracts undifferentiated or not markedly differentiated from the leaves, although in some species such as *M. genistifolium* the flowers can together form ill-defined leafy terminal spikes. Many species have a complex indument comprising multiple trichome types (often both eglandular and glandular), and in some taxa the trichomes can be branched; for example, *M. incanum* has biramous trichomes on the vegetative parts and *M. calcaratum* has stellate trichomes on the stems [20]. The calyx lobes in this clade are either prominently single-veined or the venation is obscure. *Monechma divaricatum* is notable for having only four calyx lobes with no evidence of a vestigial fifth lobe; all other species in this clade (and elsewhere in *Monechma* Group II) have five-lobed calyces. The seeds in this clade are always glabrous, usually small and compressed, with either a rounded or sharp rim and ± asymmetric in cross section, with one face being more convex than the other and often having a prominent central ridge (Figure 8H–J).

The “cleomoides” clade is notable for containing several taxonomically challenging taxa, particularly regarding three highly variable aggregate species: *M. cleomoides*, *M. divaricatum* and *M. spartiodae*. We sampled multiple accessions of the former two species. Whilst *M. divaricatum* is monophyletic, albeit with significant phylogenetic diversity, *M. cleomoides* is resolved as polyphyletic. Three accessions of that species are resolved in a clade that also contains two accessions of *M. tonsum*. These two taxa are separated primarily by differences in indumentum: *M. tonsum* has a short velvety indumentum whilst that of *M. cleomoides* usually includes ± dense mixed short and long shining trichomes (Figure 1U–W). Our results suggest that this difference in indumentum may be of limited taxonomic significance. A fourth accession of *M. cleomoides* (Klaassen et al. 2530) is resolved as sister to a clade containing *M. genistifolium* and *M. australis*, which is difficult to reconcile with the morphological evidence, in view of the fact that these two species are morphologically dissimilar to *M. cleomoides*. An AU test, however, rejects the monophyly of *M. cleomoides* and *M. tonsum* in addition to a monophyletic *M. cleomoides + M. tonsum* relationship (p < 0.001; Table 2).

4.2.4. The Status of *Monechma varians* and *Justicia platysepala*

*Monechma varians* is a rare species, confined to the Nyika Plateau of Malawi. It was recently transferred to *Justicia* sect. *Monechma* [18], although with a note that the capsule and seeds of this species had not been seen. The RADseq data place *M. varians* outside either of the two “*Monechma*” clades, it instead being resolved as sister to *J. kirkiana* in the Tylolossa clade. A specimen of *M. varians* at K, Synge WC437, was annotated by M. Hedrén in 2000, stating “a Justicia close to *J. linearispica* C.B. Clarke”. Capsule probably 4-seeded, inflorescences as in *linearispica*. We concur with this suggestion as these two species are morphologically similar, and earlier molecular phylogenetic studies have placed *Justicia linearispica* within the Tylolossa clade [13].

*Justicia platysepala* was originally described in *Monechma* on the basis of it having two- (or one-) seeded capsules. However, the seeds of this species— together with the related species *J. guerkeana* Schinz, also previously described in *Monechma* as *M. clarkei* Schinz— are tuberculate, and thus quite
unlike those of *Monechma* s.l. Our results confirm that *J. platysepala* does not belong within either of the two clades of *Monechma*.

5. Conclusions

The findings of this study confirm that the genus *Monechma* (or *Justicia* sect. *Monechma*), as previously circumscribed, represents two widely separated clades. Our findings provide insights into the evolutionary histories of these two clades. Particularly striking is the relatively recent radiation (diversifying ca. 1.9 mya) in *Monechma* Group II into the ancient deserts and xeric shrublands of the succulent biome in southwest Africa. While colonisation of the succulent biome may have involved relaxed selection on traits required to survive regular fires that are present in the savanna biome, it required adaptation to higher water deficits (evidenced by lower precipitation throughout the year) and greater extremes of low and high temperatures (higher annual temperature range). Clearly, this clade in *Monechma* Group II was able to adapt, as the radiation now accounts for more than half of the current species diversity in *Monechma* Group II despite the much longer evolutionary history of the clade within the savanna biome of tropical Africa. Our results support the need for future research to further understand the biogeographical history of these centers of biodiversity in Africa.

Given that *Justicia* s.l. is highly paraphyletic and encompasses all the taxa within the Justicioid lineage, and given the desire to avoid losing valuable taxonomic information that would be incurred through an all-encompassing *Justicia*, the only plausible option is to recognise distinct clades within *Justicia* s.l. as discrete genera and to seek morphological characters in support of these segregations. Our results show that *Monechma* Groups I and II are distinguishable from one another, albeit subtly so, by differences in the inflorescence structure and seed morphology. These two clades should therefore be elevated to generic status, and a forthcoming study will address the nomenclatural implications of recognising them as separate genera. Detailed studies employing NGS techniques, comparable to that presented in the current work, are required across the Justicioid lineage in order to delimit other genera in this complex but ecologically important plant group.

**Supplementary Materials:** The following are available online at www.mdpi.com/1424-2818/12/6/237/s1, Figure S1: Summary phylogeny of the majority-rule consensus tree from Bayesian analysis illustrating the 10 major clades of the Justicioid lineage from [13]. Within the *Harniaria* clade, species of *Monechma* Group I are sister to *Justicia odora*. Embedded within the Diclipterinae clade, species of *Monechma* Group II are sister to *Kenyacanthus nates* (*Hypocestes + Dicliptera*). Thickened branches are supported by ≥0.98 Bayesian posterior probability and ≥70% maximum likelihood bootstrap. Size of clades corresponds to the number of taxa sampled in each clade, Figure S2: The most likely phylogenetic hypothesis generated from ddRAD-seq loci from the ipyRAD de novo assembly with the minimum sample to retain a locus set to four. Asterisks [*] indicate 100% ML bootstrap and dashes [-] indicate <70% ML bootstrap, Figure S3: The most likely phylogenetic hypothesis generated from ddRAD-seq loci from the ipyRAD de novo assembly with the minimum sample to retain a locus set to 10. Asterisks [*] indicate 100% ML bootstrap and dashes [-] indicate <70% ML bootstrap, Figure S4: The most likely phylogenetic hypothesis generated from ddRAD-seq loci from the ipyRAD de novo assembly with the minimum sample to retain a locus set to 30. Asterisks [*] indicate 100% ML bootstrap and dashes [-] indicate <70% ML bootstrap. Figure S5: Phylogenetic relationships among the samples included in our study based on quartet multispecies coalescent analyses of loci resulting from the ipyRAD assembly. Numbers at nodes represent percent support across 1000 replicate quartet analyses. Asterisks [*] indicate 100% support, Table S1: Taxon, source of plant material, number of ddRAD reads per sample and number of loci per sample in our final assembly. Taxa are listed in alphabetical order by genus and species. Table S2: Results of tests for the best-fit model of evolution for biome in the ancestral state reconstruction analyses. The model in bold was selected.

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