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New insights into Trimezieae (Iridaceae) phylogeny: what do molecular data tell us?

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• *Background and Aims* The Neotropical tribe Trimezieae are taxonomically difficult. They are generally characterized by the absence of the features used to delimit their sister group Tigridieae. Delimiting the four genera that make up Trimezieae is also problematic. Previous family-level phylogenetic analyses have not examined the monophyly of the tribe or relationships within it. Reconstructing the phylogeny of Trimezieae will allow us to evaluate the status of the tribe and genera and to examine the suitability of characters traditionally used in their taxonomy.
• *Methods* Maximum parsimony and Bayesian phylogenetic analyses are presented for 37 species representing all four genera of Trimezieae. Analyses were based on nrITS sequences and a combined plastid dataset. Ancestral character state reconstructions were used to investigate the evolution of ten morphological characters previously considered taxonomically useful.
• *Key Results* Analyses of nrITS and plastid datasets strongly support the monophyly of Trimezieae and recover four principal clades with varying levels of support; these clades do not correspond to the currently recognized genera. Relationships within the four clades are not consistently resolved, although the conflicting resolutions are not strongly supported in individual analyses. Ancestral character state reconstructions suggest considerable homoplasy, especially in the floral characters used to delimit *Pseudotrimezia*.
• *Conclusions* The results strongly support recognition of Trimezieae as a tribe but suggest that both generic- and species-level taxonomy need revision. Further molecular analyses, with increased sampling of taxa and markers, are needed to support any revision. Such analyses will help determine the causes of discordance between the plastid and nuclear data and provide a framework for identifying potential morphological synapomorphies for infra-tribal groups. The results also suggest Trimezieae provide a promising model for evolutionary research.

Key words: DNA sequences, Iridaceae, Iridoideae, morphology, *Neomarica*, Neotropics, phylogenetic analysis, *Pseudiris*, *Pseudotrimezia*, *Trimezia*, Trimezieae.

INTRODUCTION

Iridaceae are a widely distributed, morphologically distinctive and species-rich family of herbaceous monocots (Goldblatt, 1990; Goldblatt *et al.*, 2008). The status and circumscription of Iridaceae have not been controversial but molecular phylogenetic analyses have had a greater impact on our understanding of evolutionary patterns within Iridaceae. These data have shown many traditionally recognized groups to be non-monophyletic and the morphological characters used to delimit them homoplastic (Reeves *et al.*, 2001; Rodriguez and Sytsma, 2005; Wilson, 2006; Goldblatt *et al.*, 2008; Petersen *et al.*, 2008).

Molecular phylogenetic trees provide the basis for the current taxonomy of Iridaceae. The seven subfamilies and ten tribes currently recognized correspond to lineages or clades identified in family-wide molecular analyses (e.g. Goldblatt *et al.*, 2008). Such studies have also shown that at a broad scale the distribution of Iridaceae is structured phylogenetically. The Neotropical Iridaceae all fall within one clade of the large subfamily Iridoideae; this clade contains the exclusively Neotropical tribes Tigridieae and Trimezieae, as well as Sisyrinchieae that has both New World and Australasian representatives (Goldblatt *et al.*, 2008). Trimezieae contain four genera and approx. 60 species. *Neomarica* Sprague (approx. 23 species) and *Trimezia* Salisb. ex Herb. (approx. 18 species) are broadly distributed in Central and South America (Chukr and Giulietti, 2008; Gil, 2012). In contrast, *Pseudiris* Chukr & A.Gil (monotypic) and *Pseudotrimezia* Foster (17 species) are restricted to the Espinhaço Range (Minas Gerais, Brazil) and Chapada Diamantina (Bahia, Brazil), respectively (Gil *et al.*, 2008; Lovo, 2012).

The status of Trimezieae as a tribe is not generally questioned, but Trimezieae lack obvious synapomorphies. Indeed in practice the tribe is characterized by the absence of the characteristics used to delimit their sister group Tigridieae (Goldblatt, 1990, 2001; Rudall, 1993). Members of Tigridieae have bulbs and plicate leaves and a chromosome base number of $x = 7$ or 14 (Goldblatt, 1982, 1990). In contrast, Trimezieae are reported to lack both these morphological features and are more variable in terms of chromosome number. Haploid chromosome numbers for Trimezieae are $n = 8–40$, implying a range of base numbers (Goldblatt, 1982, 1990; Kenton and Heywood, 1984; Chukr and

Giulietti, 2003, 2008). While these differences, especially in morphology, are useful for determining tribal affiliations they are not absolute. In each case, exceptions are known or, in the case of chromosome base number, seem likely. For example, several *Trimezia* and *Pseudotrimezia* species have plicate leaves and an underground system that resembles a bulb (Rudall, 1993). Delimiting any taxonomic group based on the absence of characters found in a related lineage is unsatisfactory. In this case, it is particularly problematic given that the same, or highly similar, states occur in Tigridieae and Trimezieae.

Delimiting generic-level groups within Trimezieae is also not without problems. The currently recognized genera are most commonly distinguished using floral morphology. The flowers of *Pseudotrimezia* differ markedly from those of the remaining genera and its generic status has been largely uncontroversial (Table 1 and Fig. 1A). The genus is characterized by similarly sized outer and inner tepals, uniformly yellow in colour and lacking prominent ornamentation. On the other hand, the flowers of *Neomarica*, *Pseudiris* and *Trimezia* are generally larger and showier (Table 1 and Fig. 1B–D, H). Although the three are easily distinguished from *Pseudotrimezia*, they have similar floral morphologies and differentiating between them is generally more difficult. In particular, there are few, if any, consistent differences in the floral morphology of *Neomarica* and *Trimezia* (Table 1). The two are most often distinguished by flowering stem shape; flat in *Neomarica* (Fig. 1E) and cylindrical in *Trimezia* (Table 1; Capellari-Júnior, 2000; Chukr and Giulietti, 2008; Gil *et al.*, 2008). However, this distinction is not clear-cut since several *Neomarica* species have nearly cylindrical stems (Ravenna, 1988*a*, 2003). Several authors (e.g. Rudall, 1993; Gil *et al.*, 2008) have also highlighted differences in the underground system. The form of the underground system in Trimezieae appears to be highly variable with descriptions suggesting it may be a corm, rhizome or bulb (Ravenna, 1981, 1988*b*; Chukr and Giulietti, 2003, 2008; Rudall, 1984, 1993; Gil *et al.*, 2008). Workers have also pointed to differences in both the orientation of this system and the persistence of the leaf bases covering it (Table 1 and Fig. 1F, G; Ravenna, 1981). The recently described *Pseudiris* is primarily distinguished from *Neomarica* and *Trimezia* by the presence of two petaloid appendages on the style (Table 1 and Fig. 1H; Gil *et al.*, 2008).

Trimezieae have received little attention in terms of molecular phylogenetic analyses. The tribe has been included in family-level studies but while these have provided insights into broader relationships, the sampling of Trimezieae has been insufficient to examine monophyly or relationships within the tribe (e.g. Reeves *et al.*, 2001; Goldblatt *et al.*, 2008). To date the only molecular phylogenetic analysis to focus on the tribe is that of Gil *et al.* (2008). This analysis suggests a monophyletic Trimezieae and implies the tribe contains several well-supported clades. While this tree was sufficient to support the recognition of *Pseudiris*, the majority of relationships within the tribe remain unresolved. A more thoroughly sampled and robust phylogenetic analysis is needed in order to evaluate the wider taxonomic issues within Trimezieae.

In the present study we explore the molecular phylogenetics of Trimezieae. We examine the monophyly of the tribe and the

TABLE 1. *Characteristics differentiating the genera of Trimezieae*

	Neomarica	*Pseudiris*	*Pseudotrimezia*	*Trimezia*
Shape of the flowering stem	Flat	Circular	Circular	Circular
Tepal size and form	Inner and outer tepals differ markedly in size, form (Fig. 1B, C)	Inner and outer tepals differ markedly in size, form (Fig. 1H)	Inner and outer tepals are similar in size, form (Fig. 1A)	Inner and outer tepals differ markedly in size, form (Fig. 1D)
Tepal colour	Shades of blue, yellow; white (Fig. 1B, C)	Blue (Fig. 1H)	Shades of yellow (Fig. 1A)	Shades of blue or yellow (Fig. 1D)
Tepal ornamentation	Outer, banding on the proximal half; inner, banding over entire surface (Fig. 1B, C)	Outer, markings absent; inner, a longitudinal yellow stripe between two white ones (Fig. 1H)	Markings usually absent from both inner and outer tepals (Fig. 1A); sometimes speckled on proximal portion	Outer, transverse banding on the proximal half; inner banding over entire surface (Fig. 1D)
Tepals with claws	Present	Present	Absent	Present
Glandular trichomes on tepals	Present	Present	Absent	Present
Style	Style apex often 2- or 3-divided forming crests; lateral appendages commonly present.	Style apex is 2-divided forming petaloid crests; lateral appendages commonly present (Fig. 1H)	Style apex never divided; no appendages	Style apex often 2- or 3-divided forming crests; lateral appendages commonly
Stigma	Transverse at base of crests	Transverse at base of crests	Apical	Transverse at base of crests
Orientation of longest axis of underground organ	Vertical or horizontal (Fig. 1G)	Vertical	Vertical (Fig. 1F)	Vertical
Persistent leaf bases covering the underground system	Present or absent (Fig. 1G)	Present	Present (Fig. 1F)	Present

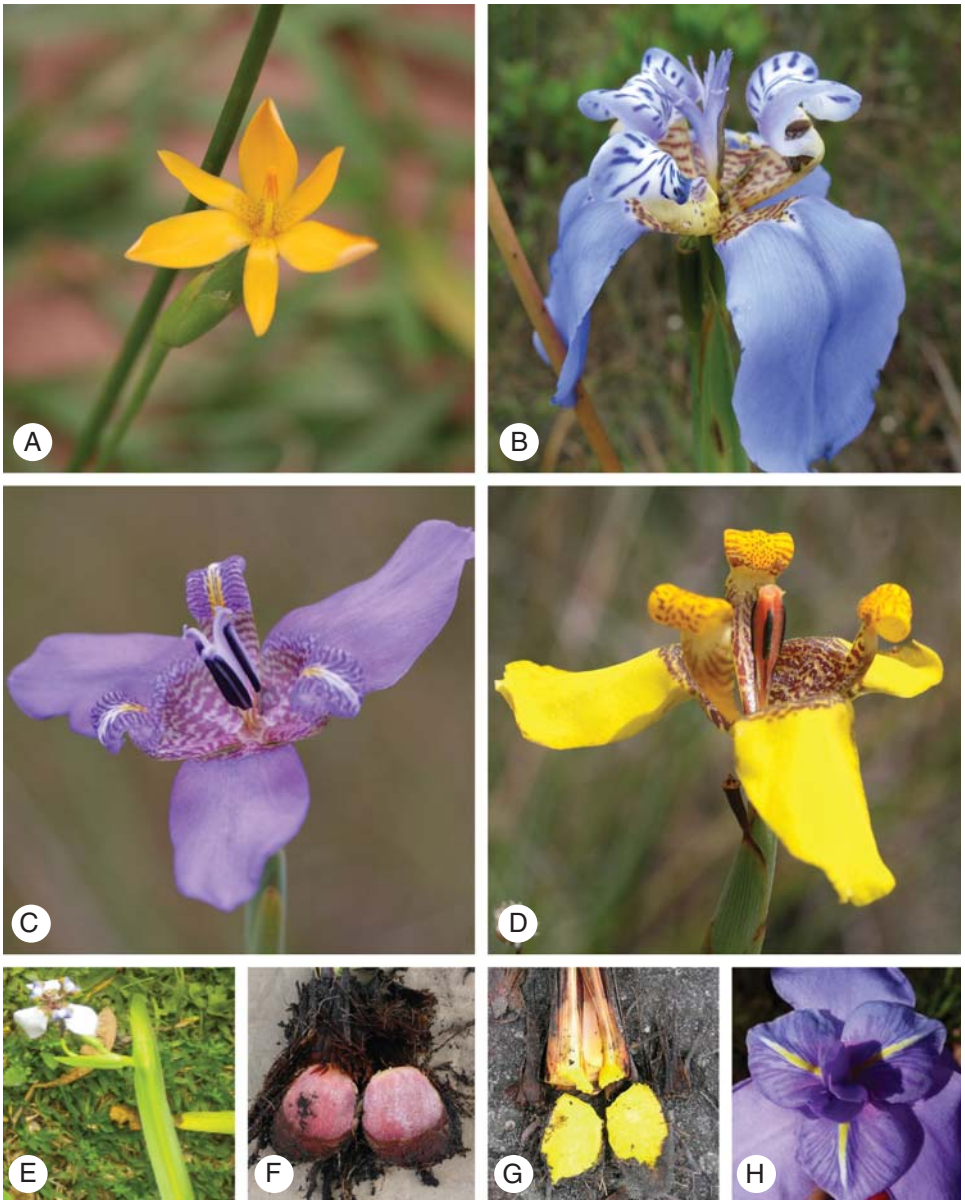

FIG. 1. Morphological diversity in Trimezieae. (A) *Pseudotrimezia synandra*, flower; (B) *Neomarica rigida*, flower; (C) *Trimezia violacea*, flower; (D) *Trimezia juncifolia*, flower; (E) *N. candida*, with flat flowering stem; (F) *P. recurvata*, underground system with persistent leaf bases (and red storage organ); (G) *N. rupestris*, underground system with persistent leaf bases (and yellow storage organ); (H) *Pseudiris speciosa* with petaloid crests on the style apices. Photographs: (A, B, E–G) J. Lovo; (C) R. Winkworth; (D) R. Mello-Silva; (H) A. Nogueira.

four currently recognized genera; we also investigate the relationships within and between these genera. Using this phylogenetic framework we evaluate patterns of evolution for ten morphological characters. These analyses provide a basis for discussing the taxonomy of the tribe and some of the characters traditionally used to delimit both the tribe and its genera.

MATERIALS AND METHODS

Taxon sampling

We included 65 accessions representing 46 species in our analyses. Sampling within Trimezieae was focused on *Pseudotrimezia*; to cover the geographical distribution and range of morphological variation, 25 accessions from 14 species were included. We sampled ten species and 11 accessions of *Neomarica*, 12 species and 18 accessions of *Trimezia* and a single accession of *Pseudiris speciosa*. Family-wide phylogenetic analyses indicate Trimezieae are closely related to tribes Tigridieae and Sisyrinchieae (e.g. Souza-Chies *et al.*, 1997; Reeves *et al.*, 2001; Goldblatt *et al.*, 2008). Here we represent Tigridieae with species of *Calydorea*, *Cipura*, *Herbertia, Gelasine* and *Kelissa*; Sysirinchieae are represented by three species of *Sisyrinchium*. The African *Dietes* (tribe Irideae) was sampled for rooting purposes. Voucher information and GenBank accession numbers for DNA sequences are provided in Table 2.

TABLE 2. *Sampled taxa with voucher information and GenBank accession numbers*

Species' name and authority	GenBank accession numbers: nrITS	*trnG* intron	*trnH-psbA* intergenic spacer	*trnK* intron	Voucher and (in brackets) herbarium codes
Sysirinchieae					
Sisyrinchium micranthum Cav.	JX070198	JX070214	JX070206	–	Lovo 238 (SPF)
Sisyrinchium aff. *palmifolium* L.	JX070199	JX070215	JX070207	–	Lovo 195 (SPF)
Sisyrinchium vaginatum Spreng.	JX070200	JX070216	JX070208	–	Lovo 126 (SPF)
Tigridieae					
Calydorea campestris (Klatt) Baker	JQ230331	JQ230501	JQ230444	JQ230388	Lovo 196 (SPF)
Cipura paludosa Aubl.	JQ230332	JQ230502	JQ230445	JQ230389	Lovo 91 (SPF)
Dietes iridioides (L.) Sweet ex Klatt	JQ230333	JQ230503	JQ230446	JQ230390	Lovo 93 (SPF)
Gelasine coerulea (Vell.) Ravenna	JQ230334	JQ230504	JQ230447	JQ230391	Calió 90 (SPF)
Gelasine coerulea	JQ230335	JQ230505	JQ230448	JQ230392	Serafim 5 (SPF)
Herbertia lahue (Molina) Goldblatt	JX070193	JX070209	JX070201	JX070217	Lovo 199 (SPF)
Kelissa brasiliensis (Baker) Ravenna	JX070194	JX070210	JX070202	JX070218	Heiden 1755 (SPF)
Trimezieae					
Neomarica caerulea (Ker Gawl.) Sprague	JX070195	JX070211	JX070203	JX070219	
Neomarica candida (Hassl.) Sprague	JX070196	JX070212	JX070204	JX070220	Lovo 241 (SPF)
Neomarica fluminensis (Ravenna) Chukr	JQ230336	JQ230506	JQ230449	JQ230393	Lovo 137 (SPF)
Neomarica gracilis (Herb.) Sprague	JQ230337	JQ230507	JQ230450	JQ230394	Melo-de-Pinna 134 (SPF)
Neomarica humilis (Klatt) Capell.	JX070197	JX070213	JX070205	JX070221	Peixoto s/n, (Mairiporã, living collection)
Neomarica imbricata (Hand.-Mazz.) Sprague	JQ230338	JQ230508	JQ230451	JQ230395	Cerqueira s/n (UEC, living collection)
Neomarica longifolia (Link & Otto) Sprague	JQ230339	JQ230509	JQ230452	JQ230396	Lovo 72 (SPF)
Neomarica northiana (Schneev.) Sprague	JQ230340	JQ230510	JQ230453	JQ230397	Gil 216 (UEC, living collection)
Neomarica rigida (Ravenna) Capell.	JQ230341	JQ230511	JQ230454	JQ230397	Lovo 191 (RB, SPF)
Neomarica rupestris (Ravenna) Chukr	JQ230342	JQ230512	JQ230455	JQ230399	Lovo 141 (SPF)
Neomarica rupestris	JQ230343	JQ230513	JQ230456	JQ230400	Lovo 210 (RB, SPF)
Pseudiris speciosa Chukr & A. Gil	JQ230344	JQ230514	JQ230457	JQ230401	Nogueira 655 (SPF)
Pseudotrimezia barretoi R.C.Foster	JQ230345	JQ230515	JQ230458	JQ230402	Mello-Silva 2680 (K, RB, SPF)
Pseudotrimezia cipoana Ravenna	JQ230349	JQ230519	JQ230462	JQ230406	Lovo 75 (RB, SPF)
Pseudotrimezia cipoana	JQ230347	JQ230517	JQ230460	JQ230404	Lovo 124 (K, NY, RB, SPF)
Pseudotrimezia cipoana	JQ230348	JQ230518	JQ230461	JQ230405	Lovo 172 (K, NY, RB, SPF)
Pseudotrimezia cipoana	JQ230350	JQ230520	JQ230463	JQ230407	Mello-Silva 2743 (K, RB, SPF)
Pseudotrimezia diamantinensis Ravenna	JQ230351	JQ230521	JQ230464	JQ230408	Lovo 107 (SPF)
Pseudotrimezia diamantinensis	JQ230352	JQ230522	JQ230465	JQ230409	Lovo 173 (K, NY, RB, SPF)
Pseudotrimezia diamantinensis	JQ230353	JQ230523	JQ230466	JQ230410	Mello-Silva 2674 (RB, SPF)
Pseudotrimezia elegans Ravenna	JQ230354	JQ230524	JQ230467	JQ230411	Lovo 144 (SPF)
Pseudotrimezia fulva Ravenna	JQ230346	JQ230516	JQ230459	JQ230403	Lovo 117 (RB, SPF)
Pseudotrimezia gracilis Chukr	JQ230355	JQ230525	JQ230468	JQ230412	Lovo 139 (SPF)
Pseudotrimezia laevis Ravenna	JQ230356	JQ230526	JQ230469	JQ230413	Lovo 108 (SPF)
Pseudotrimezia laevis	JQ230357	JQ230527	JQ230470	JQ230414	Lovo 123 (SPF)
Pseudotrimezia pauloi Chukr	JQ230358	JQ230528	JQ230471	JQ230415	Lovo 152 (K, MBM, NY, RB, SPF)
Pseudotrimezia pauloi	JQ230359	JQ230529	JQ230472	JQ230416	Lovo 176 (RB, SPF)
Pseudotrimezia planifolia Ravenna	JQ230360	JQ230530	JQ230473	JQ230417	Lovo 147 (SPF)
Pseudotrimezia recurvata Ravenna	JQ230362	JQ230532	JQ230475	JQ230419	Lovo 81 (SPF)
Pseudotrimezia recurvata	JQ230360	JQ230531	JQ230474	JQ230418	Lovo 171 (SPF)
Pseudotrimezia sublateralis Ravenna	JQ230366	JQ230536	JQ230479	JQ230423	Lovo 55 (SPF)
Pseudotrimezia sublateralis	JQ230363	JQ230533	JQ230476	JQ230420	Lovo 174 (K, NY, RB, SPF)
Pseudotrimezia sublateralis	JQ230364	JQ230534	JQ230477	JQ230421	Lovo 185 (K, SPF)
Pseudotrimezia sublateralis	JQ230365	JQ230535	JQ230478	JQ230422	Lovo 189 (RB, SPF)
Pseudotrimezia synandra Ravenna	JQ230367	JQ230537	JQ230480	JQ230424	Lovo 52 (RB, SPF)
Pseudotrimezia tenuissima Ravenna	JQ230368	JQ230538	JQ230481	JQ230425	Lovo 122 (SPF)
Trimezia brevicaulis Ravenna	JQ230369	JQ230539	JQ230482	JQ230426	Mello-Silva 2271 (RB, SPF)
Trimezia campanula Lovo & Mello-Silva	JQ230370	JQ230540	JQ230483	JQ230427	Lovo 223 (SPF)
Trimezia cathartica (Klatt) Niederl.	JQ230371	JQ230542	JQ230484	JQ230429	Lovo 42 (BHCB, HUEFS, MBM, NY, RB, SPF)
Trimezia cathartica	JQ230372	JQ230541	JQ230485	JQ230428	Lovo 178 (SPF)
Trimezia fistulosa R.C.Foster	JQ230373	JQ230543	JQ230486	JQ230430	Lovo 128 (SPF)
Trimezia galaxioides (Gomes) Ravenna	JQ230374	JQ230544	JQ230487	JQ230431	Lovo 211 (SPF)
Trimezia juncifolia (Klatt) Benth. & Hook.f.	JQ230379	JQ230549	JQ230487	JQ230436	Borges 162 (SPF)
Trimezia juncifolia	JQ230377	JQ230547	JQ230490	JQ230434	Lovo 36 (K, NY, RB, SPF)
Trimezia juncifolia	JQ230378	JQ230548	JQ230491	JQ230435	Lovo 62 (SPF)

Continued

TABLE 2. *Continued*

Species' name and authority	GenBank accession numbers				Voucher and (in brackets) herbarium codes
	nrITS	*trnG* intron	*trnH-psbA* intergenic spacer	*trnK* intron	
Trimezia juncifolia	JQ230375	JQ230545	JQ230488	JQ230432	Lovo 215 (SPF)
Trimezia juncifolia	JQ230376	JQ230546	JQ230489	JQ230433	Lovo 216 (SPF)
Trimezia lutea (Klatt) R.C.Foster	JQ230380	JQ230550	JQ230493	JQ230437	Pirani 5585 (SPF)
Trimezia martinicensis (Jacq.) Herb.	JQ230381	JQ230551	JQ230494	–	Meireles s/n (UEC, living collection)
Trimezia plicatifolia Chukr	JQ230382	JQ230552	JQ230495	JQ230438	Lovo 220 (RB, SPF)
Trimezia sincorana Ravenna	JQ230383	JQ230553	JQ230496	JQ230439	Loeuille 388 (SPF)
Trimezia spathata (Klatt) Baker	JQ230384	JQ230554	JQ230497	JQ230440	Lovo 213 (SPF)
Trimezia truncata Ravenna	JQ230386	JQ230556	JQ230499	JQ230442	Lovo 47 (BHCB, K, MBM, NY, RB, SPF)
Trimezia truncata	JQ230385	JQ230555	JQ230498	JQ230441	Lovo 115 (SPF)
Trimezia violacea (Klatt) Ravenna	JQ230387	JQ230557	JQ230500	JQ230443	Lovo 182 (K, RB, SPF)

Species' circumscriptions follow Capellari-Júnior (2000), Chukr and Giulietti (2001, 2003, 2008), Gil *et al.* (2008) and Lovo (2012).
Type species are shown in bold and dashes indicate the sequence was not recovered.

DNA extraction, amplification and sequencing

Total DNA was extracted from silica-dried leaf material (Chase and Hills, 1991) using a modified CTAB method (Ferreira and Grattapaglia, 1996).

PCR amplifications were performed in 50-μL reaction volumes typically containing 1 × PCR Buffer (Promega), 120 μM each dNTP (Invitrogen), 60 pM each amplification primer, 2 units of *Taq* DNA polymerase (Promega) and 30–50 ng of total cellular DNA. For amplification of the nrITS region reaction mixtures were supplemented with 800 μM $MgCl_2$, 3 % (v/v) bovine serum albumin and 3 % (v/v) dimethyl sulfoxide. We used the primer pairs AB101/AB102 (Douzery *et al.*, 1999), psbA/trnH^{GUG} (Sang *et al.*, 1997), 3′trnG^{uuc}/5′trnG2G (Shaw *et al.*, 2005) and trnK11/matK510R (Young *et al.*, 1999) to amplify the nrITS, *psbA-trnH* intergenic spacer, *trnG* intron and 5′ portion of the *trnK* intron (and associated portion of the *matK* gene), respectively. For the *trnK* intron we also used two novel internal primers (*PSE_matKF1* 5′-CYYGCGAGTGGACCGTTG-3′ and *PSE_matKR1* 5′-YACTTGAACCATAAGCAGG-3′), which when combined with trnK11/matK510R produced overlapping fragments. Thermocycling conditions were an initial 3 min at 98 °C, followed by 35 cycles of 95 °C for 1 min, 50–54 °C for 1 min, and 72 °C for 1 min with a final extension of 72 °C for 5–7 min. Amplification products were purified using GFX PCR purification Kits (GE Healthcare). Sequencing was performed using ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kits (Applied Biosystems, Warrington, Cheshire, UK) and analysed on either an ABI 3100 or ABI 3130XL automated DNA analyser. Sequences were assembled and edited using Sequencher (version 4·1, Gene Codes Corp., Ann Arbor, MI, USA).

Data matrices

For each locus multiple sequence alignments were produced using ClustalX 1·83 (Larkin *et al.*, 2007). Alignments were subsequently edited in MacClade 4·07 (Maddison and Maddison, 2005). Portions at the beginning and end of each alignment, as well as gapped positions with <50 % of sequences represented, were removed; we also excluded regions bordering gaps where alignments were ambiguous.

We constructed three data matrices for phylogenetic analysis; a 65-taxon plastid matrix, a 65-taxon nrITS matrix and a 30-taxon combined matrix. The plastid matrix was produced by concatenating alignments for each of the three plastid loci and the combined by adding the nrITS data to the plastid data.

Phylogenetic analyses

Maximum parsimony analyses were performed using PAUP version 4·0b10. Heuristic MP searches used TBR branch swapping, all characters equally weighted, and zero-length branches collapsed. Analyses were repeated 10 000 times with RANDOM ADDITION. Node support was estimated using a full heuristic bootstrap with 1000 replicates.

Bayesian analyses were conducted using MrBayes version 3·1·2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). Best-fit substitution models were determined for individual loci using the Bayesian Information Criterion (Schwarz, 1978) as implemented in JModelTest (Guindon and Gascuel, 2003; Posada, 2008). Searches used best-fit models (where appropriate partitioned models were used) and default settings for all other run parameters. MCMC chains were initiated with a random starting tree and run for 2×10^7 generations, sampling from the posterior distribution every 500 generations (for a total of 40 000 samples). Burn-in was determined using convergence diagnostics and trees sampled prior to stationarity eliminated.

Incongruence testing

The combinability of the three plastid loci, as well as plastid and nrITS matrices (both 65- and 30-taxon) was evaluated using the Incongruence Length Difference test (ILD; Farris *et al.*, 1994) as implemented in PAUP*4·0b10 (Swofford, 2002). Tests used 1000 partition replicates with simple taxon addition and tree–bisection–reconnection (TBR) branch swapping. ILD tests were conducted on matrices with all constant characters removed.

Incongruence between 65-taxon plastid and nrITS datasets was also evaluated using Templeton tests (Templeton, 1983). These were conducted as implemented in PAUP version 4·0b10. We used 50 % majority rule consensus topologies as rival trees for these tests; the tests were carried out using both MP and Bayesian trees.

Morphological character evolution

We investigated the evolution of ten morphological characters most of which are related to those used in previous systematic studies of Trimezieae (Table 3). In most cases we have followed Rodriguez and Sytsma (2005) when defining characters and character states. However, in several cases we have chosen to modify the characters used by previous authors. Specifically, we have defined new states for the characters related to the underground system (characters 1 and 2) and the flowering stem shape (characters 4). For example, flowering stem shape is traditionally described as either flat or cylindrical (e.g. Capellari-Júnior, 2000; Chukr and Giulietti, 2008; Gil *et al.*, 2008). We have followed Rodriguez and Sytsma (2005) in recognizing the flattened shape as winged. However, we recognize two forms: 'conspicuous wings' (wings 5 mm to 2 cm wide) and 'minute wings' (wings <2 mm wide). These correspond to flat stems and Ravenna's nearly cylindrical stems (Ravenna, 1998*a*, 2003), respectively. Stems traditionally recognized as cylindrical are here referred to as wingless. Character states were scored either from fresh material or herbarium accessions and mapped onto Bayesian trees using parsimony as implemented in McClade 4·07 (Maddison and Maddison, 2005).

TABLE 3. *Morphological characters and character states*

Characters	Character states
1. Storage in the underground system	(0) Absent, (1) present, yellow, (2) present, red
2. Persistent leaf bases covering the underground system	(0) Absent, (1) present
3. Leaf blade shape in transverse section	(0) Narrowly rhombic, (1) circular, (2) narrowly elliptic, (3) pleated, (4) linear
4. Flowering stem shape	(0) Wingless, (1) minute wings, (2) conspicuous wings
5. Ornamentation on the inner tepals	(0) Absent, (1) proximal portion speckled, (2) proximal portion with transverse bands, (3) proximal portion with longitudinal stripe
6. Glandular trichomes on the tepals	(0) Absent, (1) present
7. Orientation of the inner tepals	(0) Ascending, (1) spreading
8. Longitudinal position of the inner tepals	(0) Planar, (1) geniculate, (2) revolute
9. Crests and/or appendages on the style apices	(0) Absent, (1) present
10. Flowering time	(0) Morning, (1) afternoon

RESULTS

Sequence data and ILD tests

For 61 accessions we obtained sequences for all four of the sampled loci. For the remaining four (i.e. *Trimezia martinicensis* and the three *Sisyrinchium* species) we did not obtain useable *trnK* intron sequences; these are therefore coded as missing data in our matrices. Summary statistics for the plastid and nuclear matrices are provided in Table 4.

For the full, 65-taxon matrices, ILD tests indicate no significant incongruence between the chloroplast loci ($P = 0{\cdot}255$–$0{\cdot}437$) but a comparison of the plastid and nrITS matrices suggests significant incongruence ($P = 0{\cdot}001$). In contrast, a test on our reduced, 30-taxon matrices indicates no significant incongruence between the plastid and nrITS sequences. Summary statistics for the combined matrix are given in Table 4.

TABLE 4. *Statistics for matrices and phylogenetic analysis*

	Chloroplast	nrITS	Combined
Matrices			
No. of taxa	65	65	30
Aligned matrix length (nt)	2142 (*trnG* intron = 565; *psbA-trnH* intergenic spacer = 469; *trnK* intron = 1108)	754	2896
No. of constant sites	1801	435	2313
No. of varied sites	341	319	583
No. of parsimony informative sites	202	251	339
% informative sites	9·4	33·3	11·7
Maximum-parsimony analysis			
Tree length	481	882	1086
No. of most-parsimonious trees	14	4	30
Consistency Index	0·782	0·527	0·680
Retention Index	0·909	0·789	0·759
Bayesian analysis			
Substitution model	*trnG* intron = GTR+I; *psbA-trnH* intergenic spacer = *trnK* intron = HKY+I	K2P + I	*trnG* intron = *nrITS* = GTR + I; *psbA-trnH* intergenic spacer = HKY + I; *trnK* intron = HKY
Size of the 95 % credible tree set	72202	69252	39
Mean and variance (in brackets) of tree length	0·3622 (0·0002)	1·401 (0·0003)	0·7035 (0·0003)

Plastid analyses

Majority rule consensus trees from MP and Bayesian analyses have the same topology. In both analyses members of Trimezieae form a strongly supported clade; 99 % bootstrap support (bs) and 1·0 posterior probability (pp). Both analyses identify a set of four principal clades within Trimezieae; these clades and the relationships between them receive varying levels of support. We informally refer to these four clades as Barretoi, Fluminensis, Martinicensis and Violacea (Fig. 2).

Within Trimezieae the first branching lineage is Fluminensis (bs 51 %; pp 0·71). In our analyses this group contains seven *Neomarica* species. Within this clade relationships are for

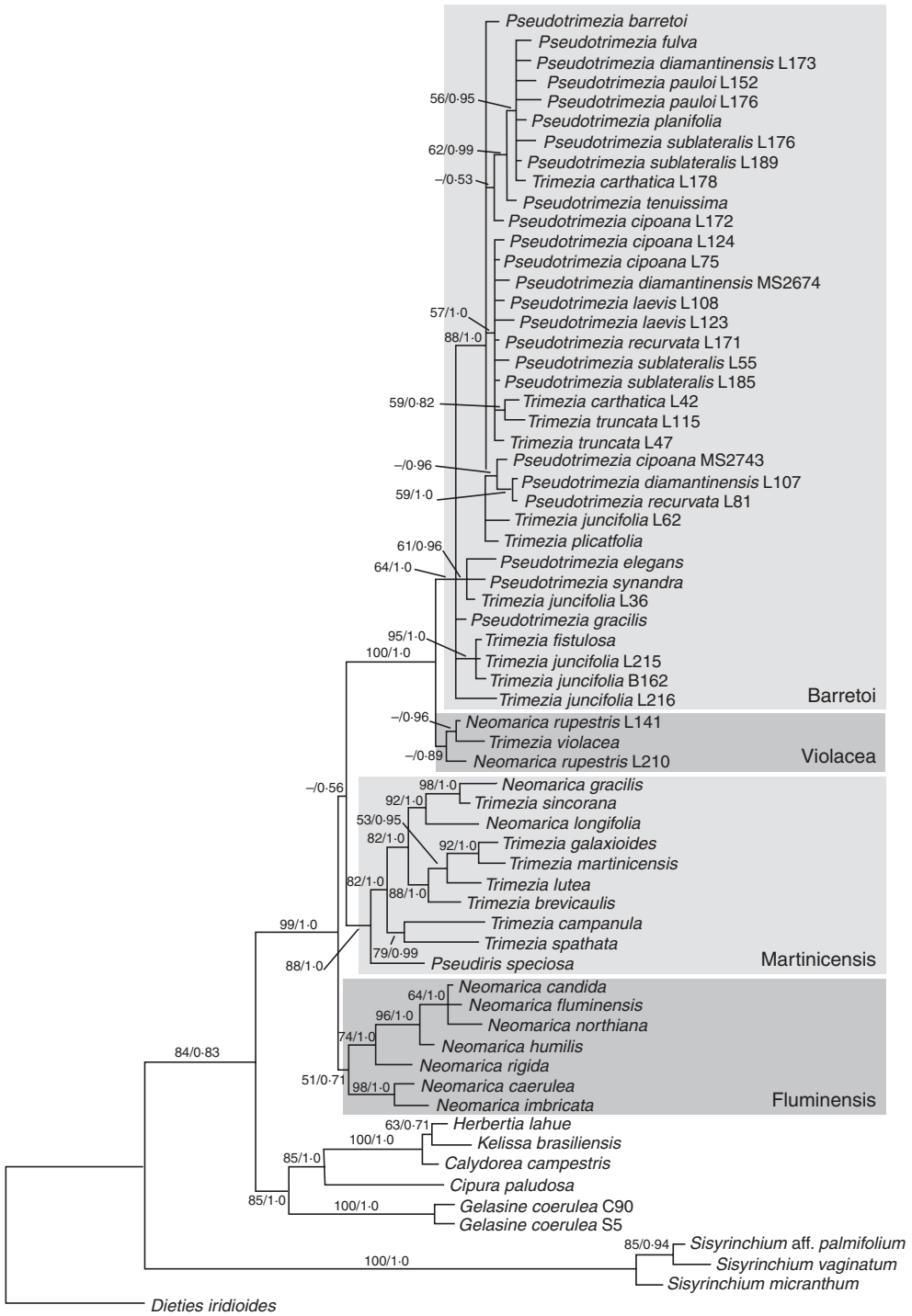

FIG. 2. Bayesian probability estimate of Trimezieae phylogeny based on 72 001 post-burn trees from analysis of the plastid dataset. Branch lengths are calculated from means of the posterior probability density. Numbers associated with branches are parsimony bootstrap percentages and posterior probabilities; a dash indicates that bootstrap support was <50 %. Clades described in the text are marked by shaded boxes and labelled on the right.

the most part well resolved and receive moderate to strong support (bs 64–98 %; pp 1·0). The next clade to diverge is Martinicensis. It includes species currently recognized as belonging to *Neomarica* and *Trimezia*, as well as *Pseudiris speciosa* (bs 88 %; pp 1·0). Within Martinicensis relationships are well resolved and often receive moderate to strong support (bs 53–98 %; pp 0·95–1·0). The Violacea and Barretoi clades together are well supported as a clade (bs 100 %; pp 1·0). The larger Barretoi clade includes representatives of both *Pseudotrimezia* and *Trimezia* (bs 64 %; pp 1·0). This clade is characterized by a number of polytomies. Violacea, containing *N. rupestris* and *T. violacea*, is the most poorly supported of the principal clades (bs <50 %; pp 0·89). Where multiple accessions of the same species are included in analyses (e.g. *N. rupestris*, *P. sublateralis*, *P. diamantinensis* and *T. juncifolia*) these do not form monophyletic groups.

nrITS analyses

Majority-rule trees for MP and Bayesian analysis of the nrITS matrix resulted in similar trees. The differences between them reflect the more limited resolution of relationships at the base of the Barretoi clade in Bayesian analyses (Fig. 3).

In terms of broad relationships, results of nrITS and combined plastid analyses are highly similar. As before the nrITS analyses indicate Trimezieae are monophyletic (bs 81 %; pp 1·0) and identify the same four principal clades (bs <50–92 %; pp 0·68–1·0). There is a single difference in the species composition of these clades. This involves the Fluminensis and Barretoi clades; *Neomarica northiana* falls in Barretoi, rather than Fluminensis, in nrITS analyses. Bayesian and MP analyses of the nrITS matrix also suggest the same relationships between the four principal clades. Support values are again somewhat variable; posterior probabilities are all 1·0 whereas bootstrap support ranges from <50–77 % (Fig. 3).

Relationships within each of the four principal clades differ between the nrITS and plastid analyses. Support for within-clade relationships in nrITS analyses varies (bs <50–100 %; pp <0·50–1·0) but in many cases the differences in resolution analyses receive at least moderate support (Figs 2 and 3). For three species (*N. rupestris*, *P. laevis* and *T. truncata*) accessions that were not monophyletic in the combined plastid analysis form clades in the nrITS tree; several others remain non-monophyletic.

Topology tests for incongruence

Templeton tests based on both MP and Bayesian topologies indicate substantial differences between the nrITS and plastid topologies. In all tests the rival trees are a significantly worse representation than the corresponding test tree (all $P<0{\cdot}0001$; $n = 72–121$).

Analyses of combined data

MP and Bayesian trees from analyses of the combined matrix are again highly similar. In this case, differences are due to more limited resolution in the Barretoi and Martinicensis clades in the MP analysis (Fig. 4).

The broad relationships suggested by the combined analyses are the same as those found in separate analyses of the nrITS and plastid matrices. Trimezieae are again monophyletic (bs 81 %; pp 1·0); the four principal clades (bs 75–100 %; pp 1·0) and relationships among them (bs 76–100 %; pp 1·0) are as in the separate analyses. Relationships in the Fluminensis and Martinicensis clades are generally well resolved and supported, whereas in Barretoi most relationships remain uncertain.

Morphological character evolution

Ancestral character state reconstructions for tepal ornamentation and leaf shape in transverse section are shown in Fig. 5. For the remaining characters reconstructions are provided in Supplementary Data Figs S1–S4. Character state reconstructions based on MP and Bayesian topologies are identical for the plastid and highly similar for the nrITS datasets; differences between the nrITS reconstructions reflect differences in resolution of the underlying trees. In contrast, reconstructions based on plastid and nrITS topologies differ with regards the distribution of character states. This is especially so in the Barretoi clade where species-level relationships differ markedly between the plastid and nrITS trees.

DISCUSSION

Similarities between nrITS, plastid and combined topologies

Phylogenetic analyses of nrITS, plastid and combined datasets indicate the same broad pattern of relationships. There is strong support for the monophyly of Trimezieae in all analyses (bs 81–99 %; pp 1·0). Within the tribe, four principal clades and relationships between them are consistently recovered, albeit with somewhat variable support. Importantly, each of the principal clades are well supported in at least one of our analyses (Figs 2, 3, 5). For example, the Barretoi clade received Bayesian pp of 1·0 in all three analyses, whereas strong MP bootstrap support for this clade is limited to analyses of the nrITS and combined data (plastid, bs 64 %). Similarly the relationships between the principal clades are consistently resolved but support is variable. This is especially so for the clade containing Barretoi, Martinicensis and Violacea; only in analyses of the combined data is this relationship more than weakly supported (bs 76 %; pp 1·0).

Support for the monophyly of Trimezieae in our analyses resolves what was previously an outstanding question in Iridaceae phylogeny. Although previous analyses were consistent with monophyly, this issue has not been examined explicitly. For example, in the family-wide analysis of Goldblatt *et al.* (2008) Trimezieae was represented by just two species; the representatives of Trimezieae were strongly supported as sister to Tigridieae with Sisyrinchieae sister to Trimezieae plus Tigridieae, again with strong support. All our analyses are consistent with this arrangement, with moderate to strong support for the pairing of Trimezieae plus Tigridieae (bs 72–84 %; pp 0·83–0·97). We think that when considered alongside the results of Goldblatt *et al.* (2008), who sampled

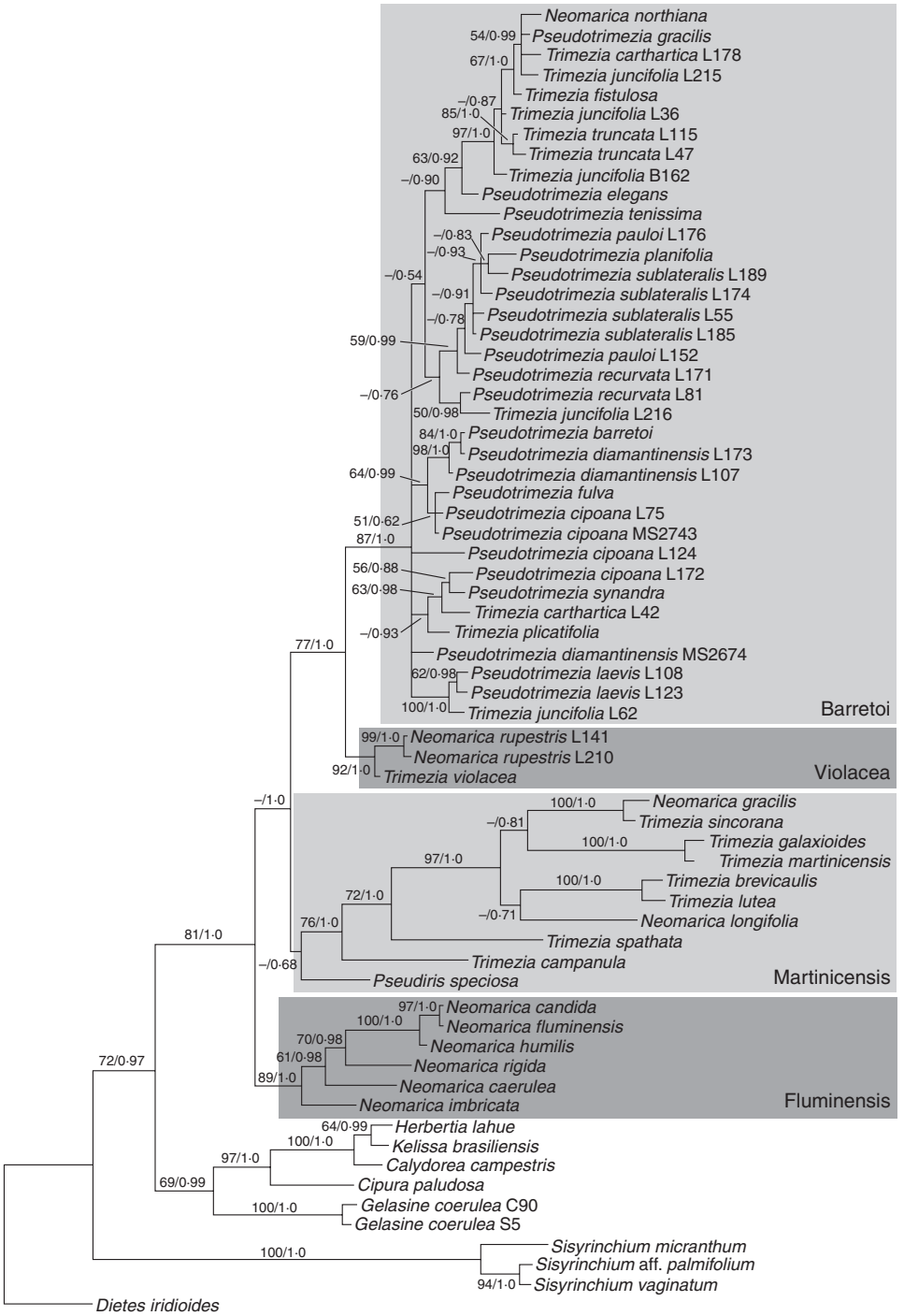

FIG. 3. Bayesian probability estimate of Trimezieae phylogeny based on 72 001 post-burn trees from analysis of the nrITS dataset. Branch lengths are calculated from means of the posterior probability density. Numbers associated with branches are parsimony bootstrap percentages and posterior probabilities; a dash indicates that bootstrap support was <50 %. Clades described in the text are marked by shaded boxes and labelled on the right.

12/15 genera of Trimezieae and 5/6 of Sisyrinchieae, our analyses strongly suggest that the traditionally circumscribed Trimezieae are monophyletic.

Our analyses indicate four principal clades within Trimezieae. However, none of these clades directly corresponds to any of the currently recognized genera. Members of the traditionally recognized *Neomarica* and *Trimezia* are each split among three clades: *Neomarica* in Martinicensis, Fluminensis and Violacea and *Trimezia* in Barretoi, Martinicensis and Violacea. This result is perhaps not entirely surprising given that Ravenna (1988*a*, 2003) found no consistent differences between these two genera and the analyses of both Rudall (1993) and Gil *et al.* (2008) had also suggested *Trimezia* was not monophyletic. Given the distinctive floral morphology of *Pseudotrimezia*

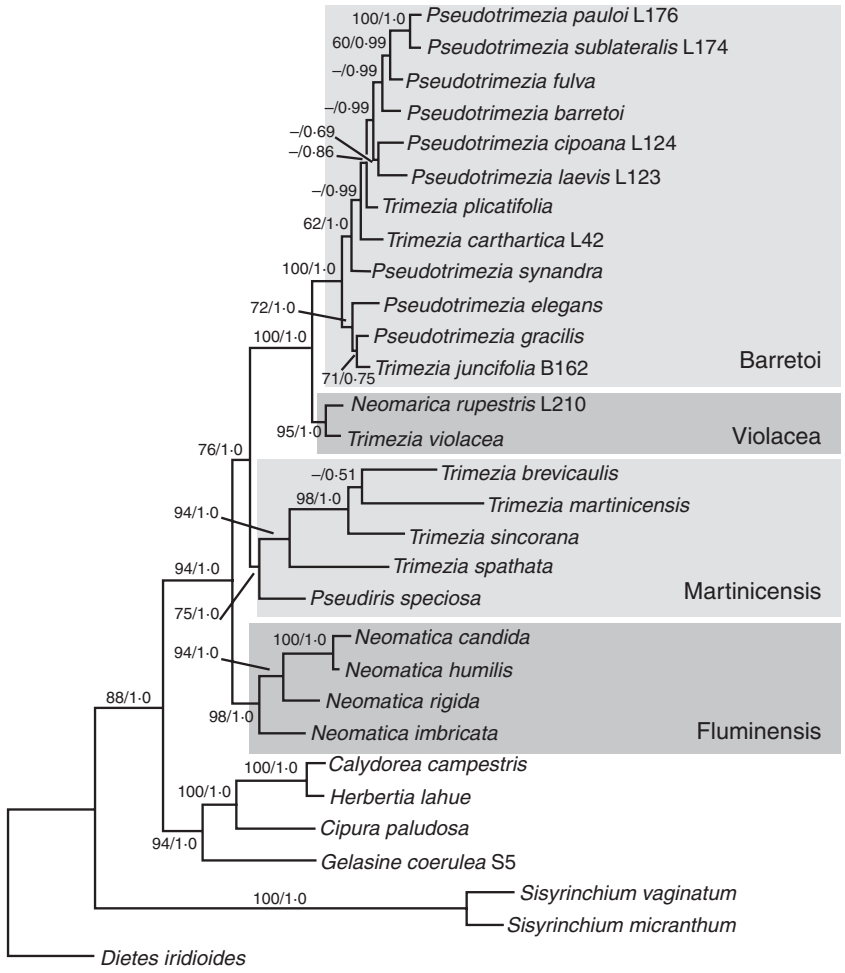

FIG. 4. Bayesian probability estimate of Trimezieae phylogeny based on 72 001 post-burn trees from analysis of the combined dataset. Branch lengths are calculated from means of the posterior probability density. Numbers associated with branches are parsimony bootstrap percentages and posterior probabilities; a dash indicates that bootstrap support was <50 %. Clades described in the text are marked by shaded boxes and labelled on the right.

the paraphyly of this genus is somewhat unexpected. The Barretoi clade includes all the included representatives of *Pseudotrimezia* plus several *Trimezia* species. Although the relationships among species-level taxa in this clade remain uncertain this result suggests that the suite of floral characteristics previously used to distinguish *Pseudotrimezia* from the remaining genera is not a good indicator of evolutionary relationships. Finally, in contrast to the results of Gil *et al.* (2008) our analyses suggest *Pseudiris* is nested in Trimezieae. Levels of support for this arrangement, at least based on the plastid and combined data, are stronger than provided by Gil *et al.* (2008) for *Pseudiris* falling sister to the remainder of the tribe. The morphological similarities linking this species to *Neomarica* and *Trimezia* appear to be a better indicator of relationships than differences suggesting it represents a novel lineage (Table 1).

Incongruence between nrITS and plastid topologies

Despite the overall similarity of plastid and nrITS trees in terms of the principal clades and their relationships to one another there is also incongruence between analyses. With one exception this discordance is limited to relationships within the four principal clades. Indeed our analyses, especially the contrast ILD test results for the 65- and 30-taxon combined matrices, suggest within-clade differences make a substantial contribution to the overall level of incongruence. Only *N. northiana* falls into different principal clades in plastid and nrITS analyses. In the plastid tree this species is a member of the Fluminensis clade but in the nrITS topology it is part of Barretoi. This result, if confirmed, has taxonomic implications since this species is the type for *Neomarica*; however, we do not think ongoing uncertainty in the placement of *N. northiana* will affect, to any great degree, the overall similarity of the plastid and nrITS trees.

In all four of the principal clades relationships between accessions differ between plastid and nrITS analyses. In the Fluminensis and Martinicensis clades some relationships are consistently resolved in the two sets of analyses. For example, the pairings of *Neomarica gracilis* with *Trimezia sincorana* and *Trimezia galaxioides* with *Trimezia martinicensis* are strongly supported by both datasets (bs 91–100 %;

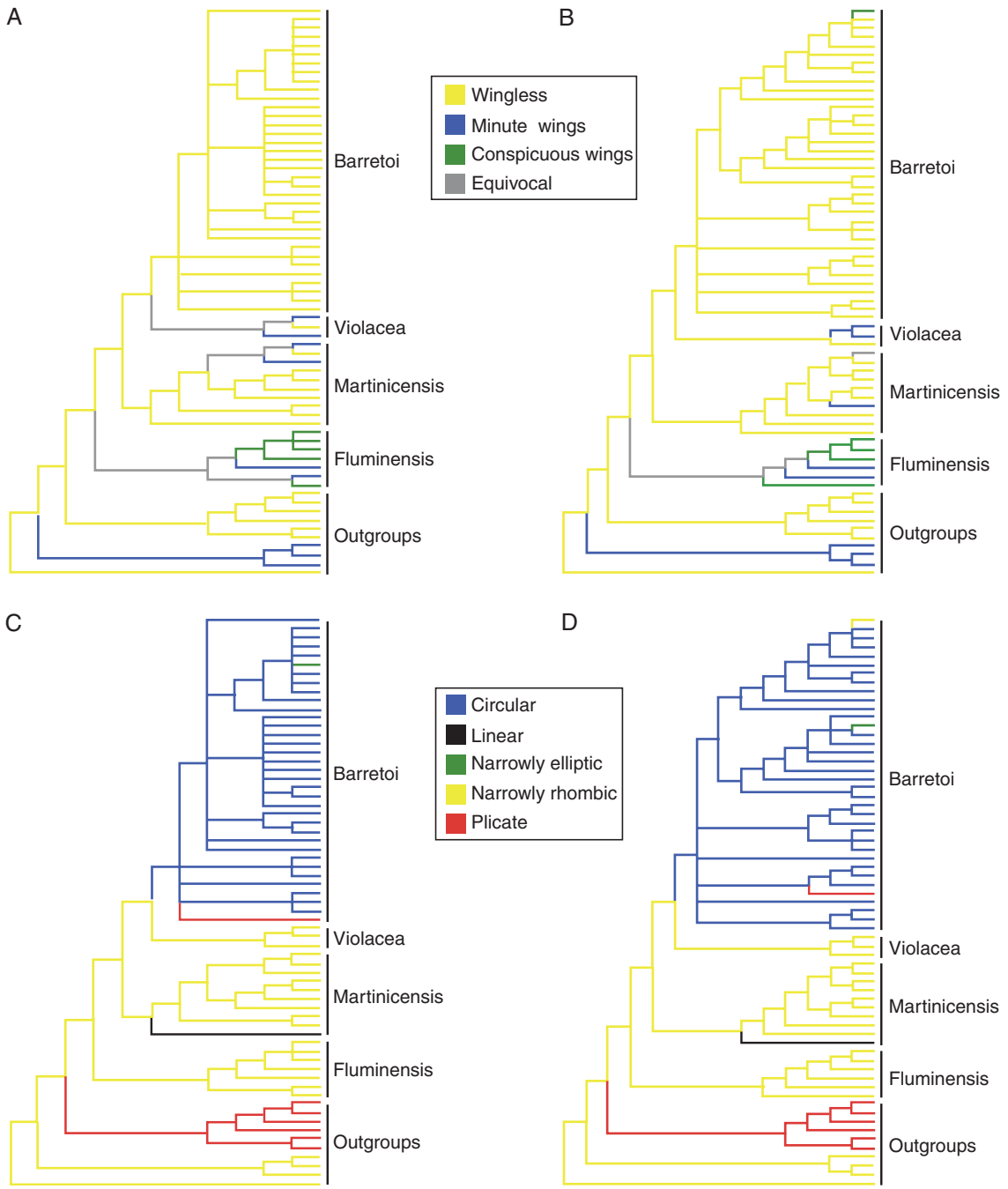

FIG. 5. Ancestral character state reconstructions for flowering stem shape (A, B) and leaf shape in transverse section (C, D). Reconstructions are based on the Bayesian probability estimate of Trimezieae phylogeny for the combined plastid (A, C) and nrITS (B, D) datasets. For clarity the species names have been omitted but clades described in the text are indicated on the right; species order is maintained relative to the phylogenetic trees shown in Fig. 2 (combined plastid) and Fig. 3 (nrITS).

pp 1·0). In Fluminensis and Martinicensis, relationships that differ between our analyses tend to receive more limited support from nrITS data. Incongruence is more widespread in the Barretoi clade where the suggested relationships differ dramatically between analyses. Even if only strongly supported nodes (bs 80–100 %; pp 0·95–1·0) are considered, none of the relationships within this clade is consistently resolved (Figs 2 and 3). The relationships of accessions representing single species also differ between plastid and nrITS. In some cases the accessions of a species are reciprocally monophyletic in nrITS but not combined plastid topologies (e.g. *N. rupestris* and *T. truncata*); in such cases the resolution suggested by the plastid data is often more poorly supported.

Limited resolution of relationships and phylogenetic incongruence pose problems from a taxonomic standpoint but they can provide important insights into the evolutionary processes that shaped contemporary diversity (e.g. Wendel and Doyle, 1998). Taken together the limited resolution and numerous

short branches in the Barretoi clade (cf. the Martinicensis clade) suggest that the morphological differentiation in this group has occurred over a relatively short period of time. Indeed, since closely related lineages may often fail to exhibit reciprocal monophyly (e.g. Funk and Omland, 2003; Syring *et al.*, 2007; Koopman and Baum, 2010) rapid diversification may also help explain why, in this clade, accessions from the same species are not monophyletic (e.g. *P. diamantinensis* and *T. juncifolia*). Typically incongruence between plastid and nuclear sequence datasets is interpreted in terms of reticulate evolution; commonly it is seen as evidence of hybridization/introgression cycles (e.g. Vriesendorp and Bakker, 2005; Drábková and Vlcek, 2010). These processes have been important in the diversification of other Iridaceae (e.g. Cholewa and Henderson, 1984; Arnold *et al.*, 1990; Arnold and Wesselingh, 2000) and in Trimezieae hybridization has been reported between Barretoi clade species (Rudall, 1993). Consistent with this latter observation, plastid and nrITS analyses suggest different relationships in the Barretoi clade, albeit in many cases with limited support (Figs 2 and 3). Our results are suggestive of underlying evolutionary processes but the present investigation is not sufficient to test specific hypotheses. This requires more detailed analyses of specific lineages.

Morphological evolution

Given differences in the underlying topologies we expect differences in the ancestral character state reconstructions based on plastid and nrITS trees. Although these differences influence the details of the individual reconstructions, for the most part they do not affect the overall patterns suggested by our analyses.

Our character state reconstructions are consistent with the observation that the traditionally recognized genera are not monophyletic. We have focused on a small selection of characters previously used to delimit taxonomic groups and, since these groups are not monophyletic, it is unsurprising that most of the characters we examined exhibit considerable homoplasy (Fig. 4 and Supplementary Data Figs S1–S4). For example, flowering stem shape (Fig. 4A, B) has commonly been used to differentiate *Neomarica* and *Trimezia*. However, our reconstructions suggest this character is not appropriate for this purpose. Winged stems (often referred to as 'flat') are restricted to *Neomarica*, but members of this genus fall within three of the principal clades; thus, the *Neomarica* species in any given principal clade are more closely related to *Trimezia* species, with wingless stems (often referred to as 'cylindrical'), from the same clade than to *Neomarica* in the other principal clades. More generally, our analyses imply that the morphological characters traditionally used to delimit generic-level taxa in Trimezieae are largely inconsistent with evolutionary relationships.

Our analyses indicate that many characters previously used in Trimezieae taxonomy are labile to change and, moreover, that there are often a limited number of possible endpoints for such change. For example, we recognize four distinct types of tepal ornamentation in Trimezieae (Table 3). Our reconstructions suggest that transverse banding was the ancestral form, with no ornamentation and speckled patterns having arisen independently on several occasions (Fig S2A, B). Similar patterns of homoplasy have previously been reported for other groups of Iridaceae. In the case of tepal ornamentation, Wilson (2006) has shown that in Iridaceae this feature is both labile to change and highly prone to parallelism.

In Trimezieae it also seems that entire character suites have been influenced in similar ways. Our analyses indicate that there have been multiple shifts between the more elaborate floral type found in *Neomarica*, *Pseudiris* and *Trimezia* and the simpler *Pseudotrimezia*-type flower. In each case the entire suite of characters associated with floral type (Table 1) has changed, apparently in unison. The lack of resolution in the Barretoi clade makes it impossible at this stage to determine whether the floral suite character associated with *Pseudotrimezia* (Table 1) arose repeatedly or just once, with several subsequent reversions to the ancestral form. Although somewhat unexpected, the repeated evolution of flower types in Trimezieae does have parallels in other groups of Iridaceae (e.g. Goldblatt, 1986; Bernhardt and Goldblatt, 2000, 2006; Goldblatt and Manning, 2006; Ikinci *et al.*, 2011; Rodriguez and Sytsma, 2005). Indeed, there are striking similarities between the *Pseudotrimezia* flower type and the '*Homeria*' type that has evolved several times in the African genus *Moraea* (Goldblatt, 1986). Both these flower types have tepals that are similar in colour, shape and position; they also both differ markedly from the more common floral forms in their respective lineages. We are currently initiating studies aimed at a more complete understanding of floral evolution in Trimezieae.

Among the previously utilized characters we found few potential synapomorphies for the principal clades. One potential exception is leaf shape in transverse section (Fig. 4C, D). Members of the Fluminensis, Martinicensis and Violacea clades share, with the exception of *Pseudiris*, the ancestral, narrowly rhombic condition. At the base of the Barretoi clade there is a shift to a circular shape. Within Barretoi only *T. plicatifolia* and *P. planifolia* do not possess this shape; as with *Pseudiris*, the character states exhibited by these two species are not found elsewhere in Trimezieae. Whether leaf shape in cross-section provides a synapomorphy for the Barretoi clade requires further study, but this result does explain why Rudall (1993) had difficulty using vegetative characters to distinguish species of *Trimezia* with leaves that are circular in transverse section from *Pseudotrimezia*. These two groups belong to the same clade.

Finally, although we have identified few synapomorphies for the principal clades, several of the characters we investigated may be useful for delimiting less inclusive groups (e.g. species or species groups). A character not previously used in Trimezieae taxonomy, but promising in this respect, is flowering time (Table 3 and Supplementary Data Fig. S4C, D). Typically, an individual flower opens for a period of just a few hours (J. Lovo, pers. obs.). Here we have classified taxa as morning or afternoon flowering, but field observations suggest additional time categories (e.g. early versus late afternoon) and these may increase the usefulness of this feature. Ultimately determining whether flowering time and other characters are useful for delimiting taxa will depend on resolving, with greater confidence, both species limits and the relationships among them.

Conclusions

Here, for the first time, we have established the monophyly of Trimezieae and examined the status of its component lineages. These results provide a number of novel insights. Perhaps most importantly is that a revision of both the generic and species level taxa is needed. Whereas broad phylogenetic relationships in the tribe are robustly resolved, discordance between plastid and nrITS trees at the within-clade level means any taxonomic changes based on the present analyses would be premature. A goal for the future is to produce a thoroughly sampled and robust phylogenetic tree that would provide the basis for a new taxonomy. Aside from the phylogenetic work, a thorough re-examination of morphological and anatomical characters is also needed, since we still lack synapomorphies for the tribe, infra-tribal groups and many species.

Beyond taxonomy a robust phylogeny for Trimezieae would provide a framework for understanding the evolutionary processes underlying the diversification of the tribe. Indeed, Trimezieae is a promising model for evolutionary research. It is a relatively small yet morphologically diverse tribe; moreover, much of this diversity is restricted to a small geographical area. This group provides a unique opportunity to study, in detail, the processes involved in speciation and morphological diversification.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Figure S1: Ancestral character reconstructions for storage in the underground system and the presence of persistent leaf bases covering the underground system. Figure S2: Ancestral character reconstructions for tepal ornamentation and glandular trichomes on the tepals. Figure S3: Ancestral character reconstructions for orientation of the inner tepals and their longitudinal position. Figure S4: Ancestral character reconstructions for appendages on style apices and flowering time.

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