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Full Length Research Paper

Phylogenetic relationships in genus *Gloriosa* L.

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In an attempt to test the monophyly of the genus *Gloriosa* L., the chloroplast region *trn*L-*trn*F sequences were employed using *Baeometra uniflora*, *Hexacyrtis dickiana*, *Neodregea glassii*, *Onixotis punctata*, *Onixotis stricta*, *Ornithoglossum parviflorum*, *Ornithoglossum undulatum*, *Ornithoglossum viride*, *Ornithoglossum vulgare* and *Sandersonia aurantiaca* as an outgroup. Results of maximum parsimony analysis reveal that *Gloriosa* is a well supported clade with the inclusion of genus *Littonia* Hook. The phylogenetic analysis resolves *Littonia modesta* Hook. as sister to the main clade of *Littonia revoilii* Franch. nested within *Gloriosa sensu stricto* species. Within the main clade, *L. revoilii* and *Gloriosa baudii* (Terracc.) Chiov. form a well supported clade. Both species are near-endemics, occurring in east Africa with *L. revoilii* extending into south Yemen. The third clade comprises species of the *Gloriosa superba* L. complex and *Gloriosa sessiliflora* Nordal & Bingham. These three lineages are also strongly supported by ecological, geographical and morphological characters.

Key words: Colchicaceae, Gloriosa, maximum parsimony analysis, phylogenetic relationships, *trn*L-*trn*F.

INTRODUCTION

Gloriosa and *Littonia*, two genera in the Colchicaceae family, have been known to be similar in vegetative and floral morphology (Baker, 1879, 1897, 1898; Buxbaum, 1937; Field, 1972; Nordenstam, 1998; Queva, 1899). The pistils of *Gloriosa* and *Littonia* are generally tricarpellate and similar (Sterling, 1975). Early workers' accurate observations and thorough diagnoses, especially on floral characteristics for both genera, perpetuated the acceptance of these generic concepts. As originally described, the genus *Littonia* included species with straight styles and connivent but not reflexed tepals, while genus *Gloriosa* was characterised by reflexed tepals, styles bent sharply at base. However, these generic distinctions became questionable and ambiguous when argued, the continued separation of the two genera in the description of *Gloriosa sessilifolia* Nordal & Bingham, a new species with intermediate characteristics between *Gloriosa* and *Littonia* (Nordal and Bingham, 1998). There is overlap in the distributions of the two genera (Figure 1); *Gloriosa* occurs in South Africa, tropical Africa, India and south-eastern Asia, while *Littonia* occurs in South Africa, tropical Africa and south Yemen.

The family Colchicaceae includes about 250 species spread across 15 genera, and is distributed in temperate to arid habitats in Africa, Asia, America, Australia and Europe. A study of the re-circumscribed and expanded Colchicaceae family has demonstrated that the biosynthesis of colchicine evolved within the common ancestor of Colchicaceae, and that this alkaloid can be regarded as a synapomorphic character for the family (Vinnersten and Larsson, 2010). As presently defined, Colchicaceae includes mainly perennial geophytes, herbs or vines arising from underground tuberous rootstock, creeping rhizomes or corms (Nordenstam, 1998). The family is monophyletic (Vinnersten and Manning, 2007; Vinnersten and Reeves, 2003), although there is need for revision of the infrafamilial classification (Manning et al., 2007). Recent work on Colchicaceae has shown strong molecular support for the monophyly of *Gloriosa* L. including *Littonia* Hook. (Vinnersten and Reeves, 2003). A molecular phylogenetic investigation using three non-coding regions (*atp*B-*rbc*L, *rps*16 and *trn*L-*trn*F) from cpDNA showed a well-supported clade (100% jacknife support) in which *Littonia* species were nested within *Gloriosa* species (Vinnersten and Reeves, 2003). Consequently, the genus *Gloriosa* has been expanded to include *Littonia* (Vinnersten and Manning, 2007),

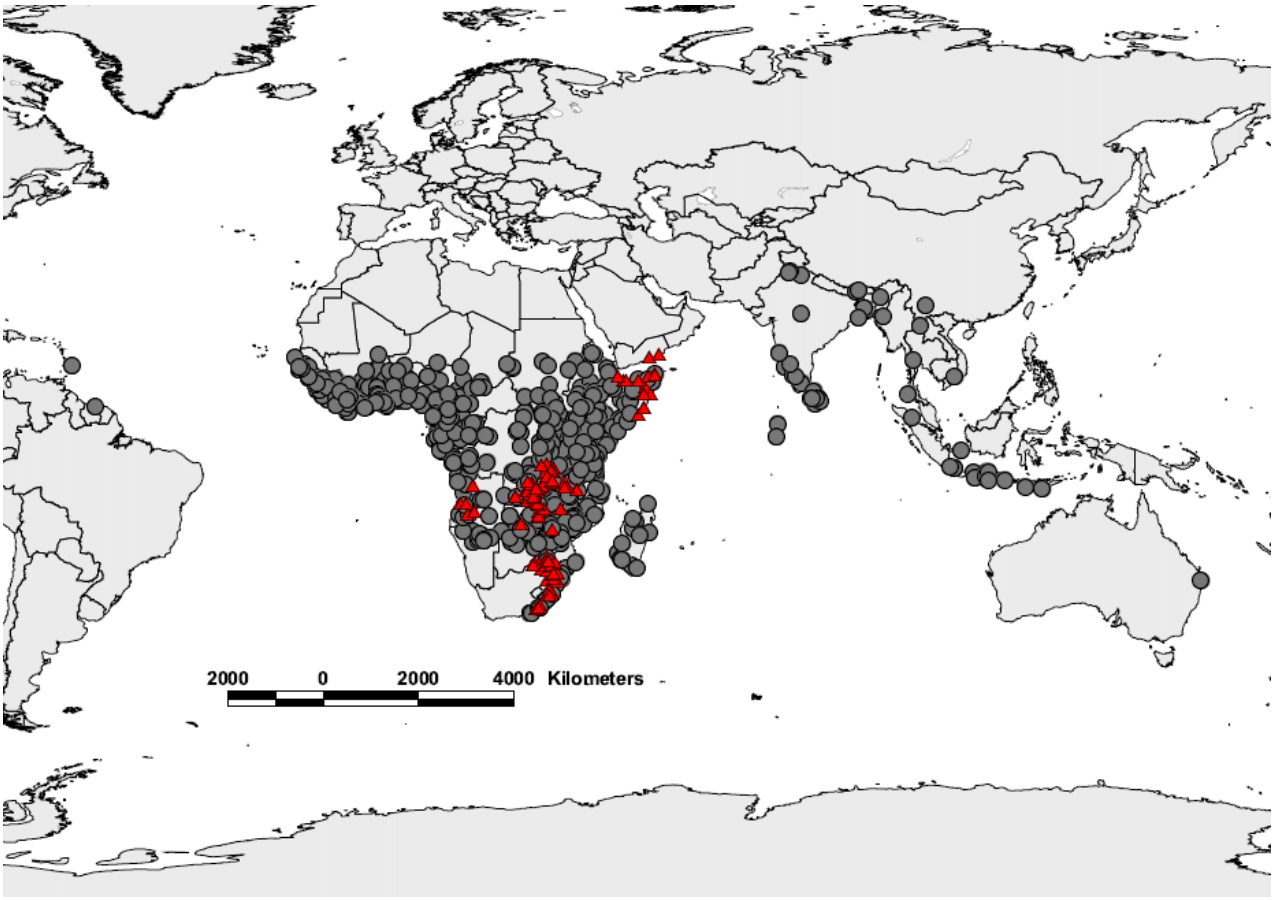

Figure 1. Distribution of *Gloriosa* and *Littonia* across their geographic ranges based on georeferenced herbarium specimens. ▲ Littonia ● *Gloriosa*.

rendering it monophyletic. Phylogenetic relationships of species within the expanded *Gloriosa* genus and their supposed allies are poorly known. The studies by Vinnersten and Reeves (2003) and Vinnersten and Manning (2007), have also brought into focus the need for further investigations to identify new synapomorphies that support the enlarged, molecular-supported *Gloriosa* genus that can provide the basis for developing a formal generic classification and taxonomic revision.

Although, the monophyly of *Gloriosa sensu lato* was demonstrated (Vinnersten and Manning, 2007; Vinnersten and Reeves, 2003), it was based on very limited sampling and does not provide a robust understanding of relationships within this clade. The generic delimitation was not properly and rigorously addressed in previous studies. Furthermore, the study did not include *Gloriosa sessilifolia*, a critical species which is morphologically intermediate between the two genera. In an attempt to address this deficiency, this study expanded the data set of Vinnersten and Reeves (2003) by augmenting the number of taxa within this clade and including *G. sessilifolia*. The current study also included morphological characters in the discussion of the resultant groups based on *trn*L-*trn*F data set. Based on previous studies by Vinnersten and Manning (2007), the genus *Littonia* is not recognised anymore; but for argument's sake, *Littonia* species are maintained in this study. The aim of this study was therefore, to further corroborate the monophyly of *Gloriosa* in light of expanded species sampling and investigate phylogenetic relationships within the expanded genus.

MATERIALS AND METHODS

DNA material, extraction, amplification and sequencing protocols

Names of all *Gloriosa* and *Littonia* species used in this study, together with their sources, voucher information and corresponding DNA extraction numbers, are shown in Table 1. Leaf material for DNA extraction and sequencing was collected in the field or obtained from herbarium specimens (Table 1). *Littonia flavovirens* Dammer, *Littonia grandiflora* De Wild. & T. Durand, *Littonia lindenii* Baker, *Littonia littonioides* (Welw. ex Baker) Krause and *Littonia rigidifolia* Bredell were not included due to lack of material suitable

Table 1. Voucher specimens and GenBank accession for sequences.

Taxon	Voucher/reference	GenBank accession number
Baeometra uniflora	Vinnersten and Reeves (2003)	AJ560294
Gloriosa baudii	Vinnersten and Reeves (2003)	AJ551359
G. carsonii	Sanane 1028 (Zambia)	New
G. sessiliflora	Bingham 12717 (Zambia)	New
G. simplex	Vinnersten and Reeves (2003)	AJ551360
G. simplex	Vinnersten and Reeves (2003)	AJ554263
G. superba	Vinnersten and Reeves (2003)	AJ551362
G. superba	Vinnersten and Reeves (2003)	AJ551361
Hexacyrtis dickiana	Vinnersten and Reeves (2003)	AJ551363
Littonia modesta	Vinnersten and Reeves (2003)	AJ551365
L. revoilii	Vinnersten and Reeves (2003)	AJ551366
Neodregea glassii	Vinnersten and Reeves (2003)	AJ551368
Onixotis punctata	Vinnersten and Reeves (2003)	AJ551369
Onixotis stricta	Vinnersten and Reeves (2003)	AJ560298
Ornithoglossum parviflorum	Vinnersten and Reeves (2003)	AJ551370
Ornithoglossum undulatum	Vinnersten and Reeves (2003)	AJ551371
Ornithoglossum viride	Vinnersten and Reeves (2003)	AJ551372
Ornithoglossum vulgare	Vinnersten and Reeves (2003)	AJ551373
Sandersonia aurantiaca	Vinnersten and Reeves (2003)	AJ560299

Table 2. Primer sequences, annealing conditions and references of the DNA regions used in this study.

Region	Primer name	Primer sequence(5'-3')	Annealing condition	Reference
*trn*L-*trn*F	C	CGAAATCGGTAGACGCTACG	30" at 58°C	Taberlet et al. (1991)
*trn*L-*trn*F	D	GGGGATAGAGGGACTTGAAC	30" at 58°C	Taberlet et al. (1991)
*trn*L-*trn*F	E	GGTTCAAGTCCCTCTATCCC	20" at 64°C	Taberlet et al. (1991)
*trn*L-*trn*F	F	ATTTGAACTGGTGACACGAG	20" at 64°C	Taberlet et al. (1991)

for molecular work. Some of the nucleotide sequences were retrieved from GenBank and have been previously published by Vinnersten and Reeves (2003). *Baeometra uniflora*, *Hexacyrtis dickiana*, *Neodregea glassii*, *Onixotis punctata*, *Onixotis stricta*, *Ornithoglossum parviflorum*, *Ornithoglossum undulatum*, *Ornithoglossum viride*, *Ornithoglossum vulgare* and *Sandersonia aurantiaea* were selected as outgroup taxa based on the results from the broad molecular systematic studies of Vinnersten and Reeves (2003).

All samples were extracted using a modified cetyltrimethyl ammonium bromide protocol (CTAB) method of Doyle and Doyle (1987). The plastid *region trnL-trnF* was amplified and sequenced using the c, d, e and f primers (Taberlet et al., 1991) as shown in Table 2. PCR reactions (25 µl) included 2.5 µl 10X Dream Taq polymerase buffer, 3.5 µl of 20 mM $MgCl_2$, 1 µl of 10 mM/ml dNTPs, 10 mM/ul of 0.5 µl forward and reverse primers, 10 mg/ml of 1 µl BSA and 1 µl of 10 mM/ml Fermentas Dream taq polymerase. PCR was performed on a PTC-200 Thermo Cycler (MJ-Research): 35 cycles; 30 s, 94°C; 1 min, 55°C; 2 min, 72°C; with an initial 4 min, 94°C; and final 7 min, 72°C. Amplification products were cleaned up using the MinElute PCR purification kit (QIAGEN), following the manufacturers' protocols. Cycle sequencing reactions were performed with the use of the BigDye Terminator Cycle sequencing kit, following the manufacturers' protocols (Zianni et al., 2006). The same primers were used for sequencing as for amplification. The products of the cycle sequencing reaction were processed in an ABI3100 capillary sequencer at the Greenomics sequencing facility. Assembly of the tracers and sequence editing were done using CodonCode Aligner (v. 3.7.1.1., CodonCode Corp., Dedham, Massachusetts) for Mac OSX.

Alignment of the three cpDNA data partitions was done by eye using Mesquite (Platt et al., 2007). Characters in parts of the sequences where alignment was ambiguous were excluded from the analyses. Individual markers were analysed under parsimony to test for incongruence (data not shown). Lack of 'hard incongruences' (conflicting nodes subject to BS > 70%; Hillis and Bull, 1993) between individual gene trees was interpreted as congruence between the data partitions, which were then combined in further analyses. For the combined analyses, a supermatrix approach was adopted, that is, including all taxa, even where data was not available for particular markers, which were coded as missing.

Maximum parsimony analyses were performed using PAUP* version 4.10b (Swofford, 2000) with the heuristic search option (TBR, ACCTRAN, MULPARS invoked). Character states were specified as unordered and equally weighted (Fitch parsimony;

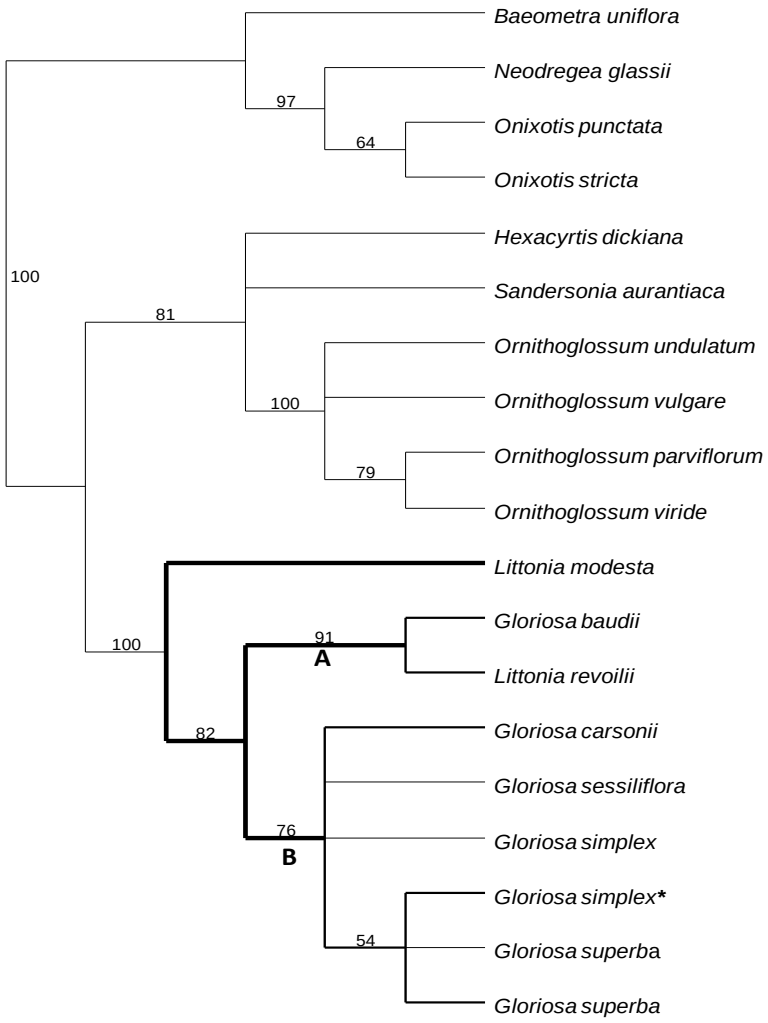

Figure 2. Maximum parsimon, 50% majority rule consensus tree based on *trn*L-*trn*F dataset. Numbers above the branches indicate bootstrap percentages based on parsimony bootstrap analysis.

Fitch, 1971). Alignment gaps were treated as described above. The search strategy consisted of 10,000 random addition sequence replicates, saving 100 trees per replicate. Clade support was assessed by non-parametric bootstrapping of the data, with 1000 bootstrap replicates, 50 addition sequence replicates per bootstrap replicate, saving 50 trees per replicate. The search options were identical as described above. When evaluating the results, we used the following descriptions of support by bootstrap values: 50 to 74% represents weak support, 75 to 84% moderate support and 85 to 100% strong support.

RESULTS

The maximum parsimony heuristic search produced 378 shortest trees with a tree length of 595 steps, with an overall consistency index (Kluge and Farris, 1969) of 0.914, and an overall retention index (RI) (Farris, 1989) of 0.877.

The maximum parsimony tree (Figure 2), shows that the ingroup species of *Gloriosa* including *Littonia* (Vinnersten and Manning, 2007; Vinnersten and Reeves, 2003) is monophyletic. The monophyly of the ingroup was maximally supported (Figure 2). The southern African species, *Littonia modesta* is resolved as sister to a clade of *Littonia revoilii* and all *Gloriosa* species, which is moderately supported (bootstrap support 82%). This clade is split into two subclades, hereafter called clade A and B. Clade A, comprising *Gloriosa baudii* (Terracc.) Chiov. and *L. revoilii*; is strongly supported with a bootstrap value of 91%. The two near-endemic species are morphologically similar and occupy overlapping geographical habitats in Ethiopia and Somalia. However, clade B has a moderate bootstrap value of 76%, and is an unresolved clade of *Gloriosa* species. *Gloriosa carsonii* Baker, *Gloriosa sessiliflora* and *Gloriosa simplex*

L. was unresolved. *G. sessiliflora*, generally regarded as a distinct species is embedded in the *Gloriosa superba* complex. Two representatives of *G. superba* and *G. simplex* formed a weakly supported polytomous clade, with bootstrap support of 54% and the relationship within this clade was unresolved.

DISCUSSION

The results of this study (Figure 2) confirmed the monophyly of the genus *Gloriosa* including *Littonia*. The results presented here support a re-circumscription of the genus *Gloriosa* in the broadest possible sense with the inclusion of *Littonia*, as was suggested by Nordal and Bingham (1998) and Vinnersten and Reeves (2003), and implemented by Vinnersten and Manning (2007). The monophyly of the genus *Gloriosa* got additional support from morphological, biochemical and chromosomal characters. Both *Gloriosa* and *Littonia* are characterised by tuberous corms (Buxbaum, 1937; Dyer, 1976; Nordenstam, 1998; Sebsebe, 1997; Thulin, 1995), their leaves frequently develop tendril-like, cirrhous tips (Dyer, 1976; Nordenstam, 1998; Queva, 1899; Sebsebe, 1997; Thulin, 1995); and colchicine occurs in all (Hegnauer, 1963; Raffauf, 1970; Vinnersten and Larsson, 2010; Wildman and Pursey, 1968). Queva (1899) also noted that crystals of calcium oxalate were lacking in both *Gloriosa* and *Littonia*. The basic chromosome number is n = 11 for *G. simplex*, *G. superba* and *L. modesta* (Bell, 1958; Cave, 1962, 1963). Sampled taxa in this study represents 58% of all known species belonging to the expanded *Gloriosa* genus, and therefore, the obtained molecular phylogeny makes it possible to hypothesise the phylogenetic relationships within the group.

The present analysis revealed some morphologically, geographically and ecologically congruent clades (Figure 2). *L. modesta* is sister to the remaining species. *L. modesta* climbs by means of leaf tendrils and has campanulate flowers. Its stems and foliage are similar to those of *G. superba* and *G. simplex*; but the flowers are different, being simpler and bell-shaped. Vegetative and floral characteristics of *L modesta* makes it resemble a South African endemic, *L. rigidifolia*. *L. modesta* is distinguished from *L. rigidifolia* by being taller and having larger leaves; and *L. modesta* being more widespread than *L. rigidifolia*, confined to the sandy areas of Waterberg, Transvaal region, South Africa. *L. modesta* is currently known from Mozambique, South Africa and Swaziland. *L. rigidifolia* would need to be sampled in further work to demonstrate whether or not morphological similarity is based on close phylogenetic relationship. *L. modesta* is the type species of the *Littonia* genus, described by Hooker (1853) differing from the genus *Gloriosa* particularly in its straight, not bent style and connivent, not reflexed tepals (Nordal and Bingham, 1998). However, it is important to note that this study did not include enough *Littonia* species to make concrete conclusions. As described in the methodology, no DNA material of the following five *Littonia* species were generated: *L. flavovirens*, *L. grandiflora*, *L. lindenii*, *L. littonioides* and *L. rigidifolia*. Expanding the sample to include some of these species confined to South Africa and south-central Africa would constitute a stronger test of the phylogenetic relationships within the *Littonia sensu stricto* species.

The second clade (B), comprises *Gloriosa baudii* and *Littonia revoilii*, and is a well supported, and geographically and morphologically coherent clade. *G. baudii* is a near-endemic taxon found in the arid regions of northern Kenya, Ethiopia and Somalia, common on sandy and stony ground. *L. revoilii* is also a near-endemic taxon, occurring in sandy and stony ground in Somalia and south Yemen. According to Sebsebe (1997) and Thulin (1995), *L. revoilii* also occurs in Djibouti and Ethiopia. Both *G. baudii* and *L. revoilii* are erect, non-climbing and usually less than 40 cm tall. Their underground stem is covered by membraneous sheathing which is usually protracted into the lowermost leaf. The two species are also characterized by linear and narrow leaves, usually less than 1.5 cm wide.

Vegetatively, *G. baudii* and *L. revoilii* are similar, they can only be distinguished using floral characters based on tepals and the style. According to Vinnersten and Manning (2007), the sister relationship between *G. baudii* and *L. revoilii* suggests that the erect tepals in *L. revoilii* are secondarily derived from the reflexed condition in *G. baudii*, and the slightly geniculate bend at the base of the style in this species may be a vestige of the sharp flexure that characterises *Gloriosa*. But this explanation does not adequately address the evolutionary relationships between *Gloriosa* and *Littonia* species considering the position of *L. modesta* which is characterised by erect tepals in the phylogenetic tree which resolves it as a sister to a clade of *L. revoilii*; and all other *Gloriosa* species (Figure 2). Occurrence of *L. revoilii* in east Africa and south Yemen has interesting biogeographical implications as it suggests a dispersal event northwards from east Africa.

Given that east Africa appears as part of the ancestral distribution of *L. revoilii* (Figure 2), the east Africa to south Yemen is the more likely route than the reverse route making the south Yemen node a possible result of a dispersal out of Africa.

Clade B is the least resolved (Figure 2), but revealed a moderately supported relationship between *G. sessiliflora* and other species of the *G. superba* complex. This result is consistent with an earlier observation made by Nordal and Bingham (1998) that *G. sessiliflora* is indistinguishable from the other forms within the *G. superba* complex in general habit. Although, *G. sessiliflora* has connivent tepals that are similar to those

of *L. sensu stricto*, the obtained phylogenetic tree suggests that it cannot be associated with this group. *G. sessiliflora* is clearly not phylogenetically distinct from the species that constitute the *G. superba* complex (Figure 2), and given the morphological diversity encompassed by *G. sensu stricto*, *G. sessiliflora* does not seem morphologically very distinct either. The only morphological character which separates *G. sessiliflora* from both *G. sensu stricto* and *L. sensu stricto* is its sessile flowers.

Species differentiation in clade B is difficult and all the species in this clade apart from *G. sessiliflora* have been regarded as belonging to *G. superba* complex (Field, 1971, 1972), a species characterized by a convoluted taxonomic history. Numerical methods utilising multivariate and univariate analyses recommended recognition of four distinct species in the *G. superba* complex: *G. baudii*, *G. carsonii*, *G. superba* and *G. simplex*. *G. superba* is the most widespread taxon, occurring in South Africa, tropical Africa and Asia. *G. simplex* and *G. carsonii* are confined to tropical Africa. *G. simplex****** examined in the present study was taken from Vinnersten and Reeves (2003) and the voucher specimen was not examined, and therefore it is cannot conclude that it is different from *G. superba*.

In conclusion, the phylogenetic analysis of the expanded *Gloriosa* genus shows several interesting relationships among its species and we still need a full understanding of the group. Given unresolved relationships and low bootstrap support for the *G. sensu stricto* clade, we need to have additional sequences from both cpDNA and nuclear DNA. This will enable us to construct a robust and better resolved phylogeny for the *Gloriosa* genus. Such further analysis might also help in understanding the *G. superba sensu stricto*, a widely distributed species stretching from South Africa, tropical Africa, Asia to south-eastern Asia.

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