MOLECULAR SYSTEMATICS OF IRIDACEAE: A COMBINED ANALYSIS OF FOUR PLASTID DNA SEQUENCE MATRICES

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ABSTRACT - Iridaceae are one of the largest families of Lilianae and probably also among the best studied families of monocotyledons. To further evaluate generic, tribal and subfamilial relationships, we have produced four plastid DNA data sets for 57 genera of Iridaceae plus outgroups: rps4, rbcL (both protein coding genes), and the trnL intron snd the trnL-F inter-gene spacer. All four matrices produce highly congruent, although not identical trees, and we thus analysed them in a combined analysis, which produced a highly resolved and well supported topology. In each of the individual trees, some genera or groups of genera are misplaced relative to Goldblatt’s and Rudall’s morphological cladistic studies, but the combined analysis produced a pattern much more similar to these previous ideas of relationships. In the combined tree, all subfamilies were resolved as monophyletic clades, except Nivenioideae, which formed a grade in which Ixioideae were embedded. The achlorophyllous Geosiris (sometimes referred to Geosiridaceae or Burmanniaceae) fell within the nivenioid grade. Most of the tribes are monophyletic, except for Ixieae, Watsonieae and Sisyrinchieae, but the topology within Ixioideae is not strongly supported due to extremely low levels of sequence divergence. Isophysis is sister to the rest of the family, and Diplarrhena falls in a well supported position as sister to Irideae/Sisyrinchieae/Tigridieae/Mariceae; Bobartia of Sisyrinchieae is supported as a member of Irideae.

KEY WORDS - Iridaceae, systematics, DNA.

INTRODUCTION

The petaloid monocot family Iridaceae comprises some 1800 species in ca. 65 genera (Goldblatt, 1990, 1991), representing one of the largest families of the superorder Lilianae (sensu Dahlgren et al., 1985). Members of Iridaceae are typically characterised by isobilateral equitant leaves, styloid crystals and flowers with only three stamens. Although worldwide in distribution, the family is centred in Africa where there are some 1000 species, most of which are restricted to southern Africa.
Rigorous and multidisciplinary studies by many authors have, to date, failed to produce a consensus sub-familial classification. The first phylogenetic classification of *Iridaceae*, using cladistic techniques (Goldblatt, 1990), formed the basis of the most recent classification of the family. This analysis used 52 characters from phytochemistry, cytology, pollen structure, anatomy and morphology to identify four major clades. Given subfamily status (Goldblatt, 1991), these were designated *Isophysidoideae*, *Nivenioideae*, *Iridoideae* and *Ixioideae*. In turn, *Iridoideae* comprised tribes *Mariceae*, *Tigrideae*, *Iridineae* and *Sisyrinchieae*, and subfamily *Ixioideae* comprised tribes *Pillansieae*, *Watsonieae* and *Ixiieae*.

In a subsequent cladistic analysis of *Iridaceae*, Rudall (1994) used 33 characters which included more anatomical characters than were used by Goldblatt (1990). This analysis recognised the four subfamilies and seven tribes *sensu* Goldblatt. However, the relationships among the subfamilies found in the two separate analyses are not identical. The principal areas of conflict concern the relationship of *Ixioideae* to the rest of the family and the placement of *Isophysis*. In Goldblatt’s scheme, *Ixioideae* were the most derived clade whereas Rudall’s analysis placed them sister to the remainder of the family. Also, Goldblatt (1990) defined *Isophysis* as the sister taxon to the rest of the family, whereas Rudall used a different outgroup and placed *Isophysis* sister to *Nivenioideae*. In Rudall’s (1994) analysis, *Isophysis* together with *Nivenioideae* then formed the most derived clade.

The most recent phylogenetic representation of *Iridaceae* is that of Souza-Chies *et al.* (1997), using molecular data derived from the region coding for protein four of the plastid small ribosomal subunit (*rps*4). This tree, inferred by the interpretation of a relatively small number of molecular characters (approximately 600 base pairs), placed *Isophysis* as the sister taxon to the rest of the family. Subfamily *Ixioideae* formed a well supported clade, although there was little resolution within it, and subfamily *Nivenioideae* did not form a monophyletic group but rather a paraphyletic grade with *Ixioideae* as its terminal clade. In this analysis the monophyly of subfamily *Iridoideae* was not supported, but there was no evidence to refute its monophyletic status. Therefore *rps*4 alone provided insufficient evidence to evaluate the monophyly of this subfamily.

Few non-molecular characters remain to be studied that could resolve the conflicts among the phylogenetic interpretations of *Iridaceae*. This study includes molecular characters from three additional plastid regions as a source of phylogenetic information and combines these data with the supplemented *rps*4 data of Souza-Chies *et al.* (1997) into a single matrix. The three plastid DNA regions sequenced are the *trnL* (UAA) intron, the *trnL-trnF* (GAA) intergene spacer (collectively known as the *trnL-F* region) and the gene for the large subunit of ribulose 1,5 bisphosphate carboxylase/oxygenase (*rbcL*). The aim of this analysis is to enhance the current understanding of *Iridaceae* phylogeny and to elucidate some presently unresolved key questions, among which the following are the most pertinent:

(i) The relationships among the four subfamilies (Goldblatt, 1991) which includes the proper placement of *Isophysis*, a Tasmanian endemic lacking one synapomorphy often assumed for *Iridaceae*, the inferior ovary. Earlier treatments had assigned *Isophysis* to its own family (Bentham and Hooker, 1883).

(ii) The familial and tribal position of the Madagascan achlorophyllous saprophyte, *Geosiris*, which in the past has been referred to *Burmanniaceae*, assigned
to its own family *Geosiridaceae* (Jonker, 1939) or placed in subfamily *Niventoideae* (Goldblatt *et al*., 1987; Goldblatt, 1990).

(iii) Correct delimitation of *Iridoideae*, including the proper status of tribe *Sisyrinchieae* within this subfamily. Of particular interest is the placement of the African genus *Bobartia* in *Sisyrinchieae* (Goldblatt & Rudall, 1992) as all other members of this tribe are American or Australasian.

(iv) Generic and tribal relationships in *Ixioideae*.

**MATERIAL AND METHODS**

Plant material and herbarium vouchers used in this analysis are listed in Table 1. Total genomic DNA was extracted from 1.0g fresh leaf or flower tissue or 0.15-0.2 g silica-dried tissue using the 2XCTAB method described by Doyle and Doyle (1987). Herbarium material of *Klattia flava* was extracted using a modified 2XCTAB method (Fay *et al*., 1997) with propan-2-ol instead of ethanol for precipitation of the DNA and a two week precipitation period at 20°C. All DNA extracts were purified by cesium-chloride ethidium-bromide equilibrium density gradients (1.55g/ml). Purified, total DNAs were dialysed in 1X TE buffer and stored at 80°C.

Three plastid regions, *rbc*L, *trn*L intron and *trn*L-F intergene spacer were amplified for the 57 species of *Iridaceae* and six outgroup taxa listed in Table 1. Those genera not represented in the *rps*4 analysis of Souza-Chies *et al*., (1997) were amplified to achieve conformity between the four data sets.

Twenty to fifty nanograms of total genomic DNA were used as a template for Taq-mediated amplification. Amplification of the *rbc*L gene was carried out using a forward primer that matched the first 20 base pairs of the exon and a reverse primer beginning at either position 1360 or 1368 on the complementary strand. Amplification using these primers produced a 1388 or 1391 base pair fragment of the *rbc*L exon. In some cases amplification of the complete gene was not possible due to degradation of the genomic DNA. In these cases the gene was amplified in two parts using internal primers 636F and 724R (this reverse primer does not work for dicotyledons). For *rbc*L four sequencing reactions per taxon were required with primers 1F, 636F, 724R and 1360R/1368R (Muasya *et al*., 1998). In most cases greater than 80% overlap was achieved.

Primers «c» and «f» (Taberlet *et al*., 1991) were used to amplify the intron and intergene spacer region between the *trn*L 3' and *trn*F exons. The amplified fragment varied in length from approximately 650 to 900 base pairs and resulted in an aligned matrix of 1250 base pairs. For cases in which complete amplification of the «c» to «f» region failed, internal primers «d» and «e» (Taberlet *et al*., 1991) were used to amplify the gene in two non-overlapping segments. Only two sequencing reactions, with primers «c» and «f», were required in cases for which complete amplification of the *trn*L-F region was successful. Greater than 80% overlap was achieved in most cases. All *trn*L-F sequences were easily aligned by eye. Four discrete gaps were also coded as 0/1 characters, otherwise gaps were coded as missing.

A fragment including the *rps*4 gene, an intergene spacer and the *ser*-tRNA gene was amplified using primers *rps*5' and t RNAS (Souza-Chies *et al*., 1997). The resulting amplified fragment was approximately 800 base pairs in length. Only the 600bp *rps*4 exon was used in this analysis. As for *trn*L-F, all *rps*4 sequences were aligned by eye.
### TABLE 1

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  Goldblatt & Manning 9672B (I-183), MO

  Galaxia sp.
  Goldblatt & Nanni 10254 (I-228), MO

  Gynandriris sisyrinicum Parl.
  M.W. Chase (I-107), K

Subtribe Iridinae
  Dietes robinsoniana Klatt
  Pickard 3377 (I-8), MO

  Dietes robinsoniana Klatt
  MNHN (S 88-82)

  Iris unguicularis Poir.
  M.W. Chase (I-100), K

  Belamcanda chinensis Adans.
  MNHN (IA 84-1389)

Subtribe Ferrariinae
  Ferraria crispa Burm.
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  M.W. Chase (I-242), K

  Tigridia sp. Juss.
  MNHN (EB)

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  Castillo (I-202), MO

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  Porto Alegre (027)

  Cipura campanulata Ravena
  Henrich 143 (I-201), MO

  Eleuthereine latifolia (Standl.& Will.) Ravena
  Goldblatt 9072 (I-204), MO

  Ennealophus euryandrus (Gris.) Ravena
  Solomon 9972 (I-23), MO

  Gelasine elongata (R.Grah.) Ravena
  Goldblatt 5925 (I-48), MO

Souza-Chies et al.,(1997)
Souza-Chies et al.,(1997)
Souza-Chies et al.,(1997)
Souza-Chies et al.,(1997)
Souza-Chies et al.,(1997)
Souza-Chies et al.,(1997)
**Calydorea pallens** Griseb.  
**Herbertia pulchella** Sweet  
**Alophia verucruzan**a Goldbl. & T.M. Howard  
**Hesperoxiphion peruvianum** Bak.  

**Tribe Mariceae**  
**Neomarica northiana** Sprague  
**Neomarica sp.** Sprague  
**Trimezia martincensis** (Jacq.) Herb.  
**Trimezia stayermarkii** R. Foster  

**Subfamily Ixioideae**  
**Tribe Pillansieae**  
**Pillansia templemanni** L. Bolus  

**Tribe Watsonieae**  
**Lapeirousia neglecta** Goldbl.  
**Savannosiphon euryphylla**  
**Micranthus junceus** N.E. Brown  
**Thereianthus racemosus** (Klatt) Lewis  
**Watsonia anguta** Ker Gawl.  

**Tribe Ixieae**  
**Ixia latifolia** D. Delaroche  
**Chasmanthe aethiopica** (L.) N.E. Br.  
**Freesia alba** Bak.  

Goldblatt 9579 (I-200), MO  
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Goldblatt (I-206), MO  
Solomon 6950 (I-207), MO  
Porto Alegre (010)  
Berry 3802 (I-5), MO  
Goldblatt 1245 54, MO  
Bean (I-6), MO  
Goldblatt & Manning 9489 (I-7), MO  
Bolnick (I-193), MO  
M.W. Chase (I-156), K  
Goldblatt 10454 (I-224), K  
Goldblatt 6904 (I-4), MO  
Goldblatt & Manning 9594 (I-184), MO  
Goldblatt 5293 (I-68), MO  
S. Chicos et al. (1997)  
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All gaps were coded as missing.

Amplified double-stranded DNA fragments were purified using «Wizard» mini columns (Promega) and directly sequenced on an ABI 373A automated sequencer using standard dye-terminator chemistry following manufacturers protocols (Applied Biosystems Inc.). For editing and assembly of the complimentary strands «Sequence Navigator» and «Autoassembler» (Applied Biosystems Inc.) were used.

All cladistic analyses were performed using the parsimony algorithm of the software package PAUP for Macintosh (phylogenetic analysis using parsimony version 3.1.1; Swofford, 1993) on a Power Macintosh 7200/90 with 16MB RAM. The data matrices corresponding to each of the four plastid DNA regions and a combined data matrix of all four were analysed using 1000 replicates of random taxon addition order, tree bisection-reconnection (TBR) branch swapping, with MULPARS on, and all character transformations treated as equally likely (Fitch parsimony; Fitch, 1971). To minimise the time spent searching sub-optimal «islands» (Maddison, 1991), a limit of ten trees were saved from each replicate. Characters were reweighted according to their corresponding RC values and after each round of reweighting a heuristic search with simple taxon addition was performed. When the tree length remained the same in two successive rounds these were the finite trees. The final successive (SW) weights were then applied to either all trees or a random large subset (more than 2000) of the initial trees collected during branch swapping for a second heuristic search with random taxon addition TBR branch swapping and MULPARS on. Internal support was assessed using 1000 bootstrap replicates (Felsenstein, 1985) and the SW weights. Only those groups of greater than 50% frequency were reported.
RESULTS

Since all analyses produced highly congruent patterns, we present here only the best supported, the combined analysis. The combined analysis of the three plastid DNA regions included 3232 characters. Parsimony analysis with equal weights produced 244 equally parsimonious trees of 2464 steps with consistency index (CI) = 0.58 and retention index (RI) = 0.73. Applying successive weights resulted in three equally parsimonious trees of 1021582 steps with CI = 0.86 and RI = 0.90. These trees were a subset of the Fitch trees (i.e. they also had 2464 steps).

The combined tree demonstrates excellent support for subfamily Ixioideae, however several polytomies occur within the subfamily. The delimitation of tribes Watsonieae, Pillansieae and Ixieae sensu Goldblatt (1990) are not supported. However, some groupings are well resolved with high bootstrap support, notably the alliance of two genera of Watsonieae, Thereianthus and Micranthus, with the monogeneric subfamily Pillansieae. Watsonia and Lapeirousia (Watsonieae) form a supported clade, as do three genera of Ixieae, Schizostylis, Hesperantha and Geissorrhiza. Freesia and Anomatheca (Ixieae) are also well supported as sister taxa. Ixioideae form the most derived clade with a paraphyletic Nivenioideae. Within this paraphyletic grade three genera, (Witsenia, Klattia and Nivenia) form a monophyletic group. Iridoideae are monophyletic, with tribes Irideae, Tigridieae, Mariceae and Sisyrinchieae resolved in all trees. However, Bobartia and Diplarrhena (both often placed in Sisyrinchieae), occupy positions outside Sisyrinchieae. Bobartia is sister to Irideae, and Diplarrhena is sister to the rest of Iridoideae. Isophysis is the sister taxon to the rest of the family.

DISCUSSION

Previous cladistic analyses of non-molecular characters (Goldblatt, 1990; Rudall, 1994) have identified four distinct groups within Iridaceae. To date, no evaluation has been performed to evaluate the robustness of these clades and hence the two phylogenies represent somewhat conflicting hypotheses which must be equally accepted as possible explanations of Iridaceae phylogeny. In contrast, the tree presented here has been evaluated for the level of support attributable to each of the individual clades. The taxonomic implications of the combined tree are discussed below.

Affinities of the monotypic genus Isophysis vary considerably among classification systems, and its inclusion in Iridaceae is controversial largely due to its possession of a superior ovary. Isophysis does, however, share with the rest of the family at least two synapomorphies: presence of stylloid calcium oxalate crystals (Goldblatt et al., 1984) and flowers with three stamens. These characters have led recent authors to include Isophysis in Iridaceae: tribe Isophysideae (Hutchinson, 1934) and subfamily Isophysidoideae (Goldblatt et al., 1984; Dahlgren et al., 1985). The precise placement of Isophysis within Iridaceae has also been disputed (Goldblatt, 1990; Rudall, 1994). In the combined molecular analysis Isophysis belongs in a position as sister to the remainder of Iridaceae, as Goldblatt (1990) suggested. Isophysis is unambiguously placed in all combined trees, and bootstrap support for the Iridaceae clade excluding Isophysis is high.
Figure 1 - One of the three most parsimonious trees found with successive weighting. Fitch lengths are shown above the branches. Bootstrap values achieved with successive weights and with Fitch weights (underlined) are shown below the branches. The taxonomic scheme is that of Goldblatt (1990).
Figure 1 (cont)
The placement of *Isophysis* as sister to *Iridaceae* in Goldblatt’s analysis (1990) may be explained somewhat by his choice of outgroups which all possess a superior ovary. If *Isophysis* is the earliest diverging genus of *Iridaceae*, this could imply that the superior ovary is the ancestral state for the family. Subsequent to Goldblatt’s analysis, the rbcL monocot analysis (Chase *et al*., 1995) placed *Iridaceae* within the ‘lower’ asparagoids in a grade including *Doryanthaceae* and *Ixioliriaceae*, with *Tecophilaeaceae* (including *Cyanastraceae*) as their sister group. These closest relatives, as implied by the rbcL tree, all possess an inferior ovary and are the families from which outgroup taxa have been chosen for this molecular analysis. Therefore, since both ingroup and outgroup taxa possess an inferior ovary, the superior ovary of *Isophysis* must be regarded as an autapomorphy and is thus uninformative.

The six genera included in *Nivenioideae sensu* Goldblatt (1990) are represented in this analysis. The monophyly of *Nivenioideae* is not supported in any of the molecular analyses; instead the subfamily comprises a paraphyletic grade which collectively forms a clade with subfamily *Ixioideae*. Within paraphyletic *Nivenioideae*, the three shrubby Cape genera *Witsenia*, *Klattia* and *Nivenia* form a well supported clade (100% bootstrap) in the combined analysis. The inclusion of the Madagascan saprophyte *Geosiris* in the *Nivenioideae-Ixioideae* clade is consistent in all of the molecular analyses and confirms its proper status within, and as a member, of the family (Goldblatt *et al*., 1987). In the combined tree, the Australian genus *Patersonia* represents the sister group of the *Nivenioideae-Iridoideae* clade.

Coherence of *Nivenioideae* as a monophyletic group has been questioned by previous authors (Goldblatt, 1990) because, in addition to its broad geographical distribution, only three non-molecular characters define the subfamily: binate rhipidia, a blue perianth, and a fugacious flower. The last two are also found in *Iridoideae*, and none of these characters can be assessed robustly by outgroup comparison.

The delimitation of the largely African subfamily *Ixioideae* is in accordance with most systems of classification of *Iridaceae* which have consistently accepted its existence as a distinct group within the family (tribe *Ixiieae* of Bentham and Hooker, 1883; Diels, 1930). *Ixioideae* are well defined by both morphological and anatomical characters, but relationships within this subfamily remain ambiguous due to the lack of divergence demonstrated by the plastid DNA regions used in this analysis. Several sub-familial groupings do emerge in the combined analysis. Tribe *Watsonieae* are split into two well supported groups with the exception of *Savannosiphon*. One of these groups, comprising *Theiranthus* and *Micranthus*, appears to be associated with the monogeneric tribe *Pillansieae*. These three taxa are embedded in the partly unresolved tribe *Ixieae*.

Sampling for *Ixioideae* may be improved but it does appear that the plastid regions used to reconstruct this phylogeny do not exhibit enough variation to resolve the relationships within this apparently rapidly radiating subfamily. Normally, improved resolution may be achieved by sequencing more variable nuclear DNA regions, for example the internal transcribed spacer region (ITS). However, within many genera of *Iridaceae*, ITS rDNA appears to exist in a series of highly divergent repeats at different chromosomal locations (Chase *et al*., unpubl.). This makes the ITS region difficult to use for phylogenetic reconstruction.
Subfamily Iridoideae sensu Goldblatt (1990) emerges as a monophyletic group in the combined analysis with bootstrap support of 99%. Many tribal groupings are also well resolved and supported, and in the main part are in accordance with those outlined by Goldblatt (1990). The exceptions to the tribal groupings sensu Goldblatt (1990) are the placement of two members of tribe Sisyrinchieae: Bobartia and Diplarrhena. In Goldblatt’s scheme, Bobartia is the only South African member of the tribe, and molecular data place Bobartia in a clade with representatives of the South African tribe Irideae, a position which has been previously considered but not supported by any prior cladistic analysis (Goldblatt & Rudall, 1992). Diplarrhena is unusual within Iridaceae as it only possess two stamens whereas three stamens is uniform for the rest of the family (Rudall and Goldblatt, this volume). The combined molecular analysis positions Diplarrhena as sister to the remainder of Iridoideae.

Based upon the combined parsimony analysis of the three plastid DNA regions the following taxonomic recommendations are appropriate:

(i) Combination of Nivenioideae and Ixioideae into a larger Ixioideae with tribes Patersonieae, Geosirieae, Aristeae, Nivenieae (including Nivenia, Witsenia and Klattia) and Ixieae (including all the current Ixieae, Watsonieae and Pillansieae). Since only fairly insignificant characters delimit the groupings in the present Ixioideae there is no strong argument for recognition of tribes Watsonieae and Pillansieae.

(ii) In subfamily Iridoideae Diplarrhena, as a distinct species within the group, should be assigned to its own tribe Diplarrheneae (Rudall and Goldblatt, this volume). Bobartia should be included in tribe Irideae rather than Sisyrinchieae.

In conclusion, this study strongly supports combining data for systematic inference when more than one data set is available. Combining consensus trees would not resolve the positions of Isophysis, Diplarrhena and Patersonia for example. Further work should include the combination of non-molecular characters with DNA sequence data as this may provide greater resolution within Ixioideae in particular.

REFERENCES


