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Floral and macroecological evolution within *Cyrtanthus* (Amaryllidaceae): Inferences from combined analyses of plastid *ndhF* and nrDNA ITS sequences

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Abstract

One of the most diverse members of Amaryllidaceae is *Cyrtanthus* Aiton, a large, sub-Saharan Africa genus of approximately 55 species found mostly in South Africa. To investigate phylogenetic and biogeographic relationships within *Cyrtanthus*, sequence data from the plastid *ndhF* gene and the ITS nrDNA region for 41 species were analyzed with parsimony, maximum likelihood, and Bayesian-inference approaches. Various recombination detection algorithms were used to test for interspecific hybridization in the ITS alignment. The genus resolved as monophyletic, comprising three poorly to well-supported major lineages: a predominantly Afrotemperate lineage, largely restricted to seasonally moist sites in summer rainfall southern Africa, a subtropical lineage found mostly in nonseasonal rainfall regions, often in dry habitats, and a Cape Floristic Region-centered lineage in which most species are concentrated in the summer-dry to nonseasonal rainfall southwest. The ITS sequence alignment shows no evidence for reticulation between any of the species. Relationships inferred by the molecular data disagree with those derived from morphological data, but agree with previously published groupings based on karyotype morphology. Fitch optimization of selected floral characters on the combined gene tree reveals recurrent patterns of convergence. Ornithophilous floral forms occur in parallel among the three primary clades, putatively sphingophilous species are concentrated in the Afrotemperate lineage in seasonally moist upland grasslands; the brush-type *Aeropetes tulbaghia* butterfly and inferred long-proboscid fly pollination syndromes are unique in the Cape lineage. Macroecological factors inferred to have influenced the evolution of *Cyrtanthus* are changes in rainfall seasonality, the advent of fire, and the availability of new habitats at high and low altitudes and in rock-free soils or rock crevices. This study gives greater clarity on relationships within the genus and enables its division into three informal infrageneric groups.

Keywords: Africa; Amaryllidaceae; *Cyrtanthus*; Floral morphology; Molecular systematics; Monocotyledons

1. Introduction

The genus *Cyrtanthus* Aiton is endemic to sub-Saharan Africa, with well over 90% of its species concentrated in South Africa (Dyer, 1939; Reid and Dyer, 1984). With about 55 species it is the largest genus of southern Africa's Amaryllidaceae (Snijman and Archer, 2003) and one of the largest in the family overall. Within this ecologically diverse region *Cyrtanthus* extends from the summer-dry southwest to the summer rainfall northeast. A remarkable response of several species within fire-prone ecosystems is their dependence on fire to flower (Gordon-Gray and Wright, 1969; Le Maitre and Midgley, 1992; Keeley, 1993).

Traub (1963) placed *Cyrtanthus* in its own tribe, Cyrtantheae, a treatment maintained by Meerow and Snijman (1998), whereas Müller-Doblies and Müller-Doblies (1996) placed *Cyrtanthus* in the tribe Haemantheae, albeit as a monotypic subtribe, Cyrtanthinae, based on bulb morphology and chromosome number, a classification accepted by Dahlgren et al. (1985). Combined *rbcL* and plastid *trnL-F* sequences (Meerow et al., 1999) indicated a position for *Cyrtanthus* as sister to the remainder of Amaryllidaceae after the branching of the tribe Amaryllideae. Plastid *ndhF* sequences (Meerow and

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Snijman, 2006), however, resolved *Cyrtanthus* as sister to a clade comprising Calostemmateae and Haemantheae. An ITS alignment across the entire family (Meerow, unpubl. data) places *Cyrtanthus* as sister to Haemantheae, but until additional data are available the position of the genus relative to the rest of the family remains ambiguous.

The genus exhibits a high level of floral morphological diversity which is unparalleled in any other genus of the family. Conversely, the genus shows great consistency in chromosome number, with $2n=16$ characteristic of most, if not all, of the species (Wilsenach, 1963; Ising, 1970; Strydom et al., 2007). It is also the only African genus with the flattened, winged, phytomelanous seed, so common in the American clade of the family (Meerow and Snijman, 1998). Following Baker's (1888, 1896) treatments of *Cyrtanthus*, Dyer (1939) provided a synoptic review of the genus and Nordal (1979) revised the two East African species. The most recent account is that of Reid and Dyer (1984); six new species have since been described (Hilliard and Burtt, 1986; Snijman and Van Jaarsveld, 1995; Snijman, 1999, 2001, 2003, 2007).

The showiness of the flowers in *Cyrtanthus* is comparable to those of Orchidaceae and Iridaceae in southern Africa, families for which extensive data on pollination systems are available (Goldblatt et al., 1995, 1998; Johnson et al., 1998; Linder and Kurzweil 1999; Goldblatt and Manning, 1999, 2000). These studies indicate that shifts in pollination systems occur frequently within groups of closely related species and have necessitated the revision of several genera formerly classified on floral similarities (Goldblatt and Manning, 1998, 2007).

Using plastid *ndhF* and nrDNA ITS sequences, this study explores the phylogeny of *Cyrtanthus*. We use the phylogeny to examine ancestral habitats and distributions within the lineage and we test whether floral morphology is congruent with the phylogeny generated by the molecular data. By means of floral types (Vogel, 1954; Faegri and Van der Pijl, 1979; Johnson and Bond, 1994; Goldblatt and Manning, 2006) we infer the pollinators of species, trace the evolution of selected floral characters and assess the taxonomic implications for the genus.

2. Material and methods

2.1. Sampling

Plastid *ndhF* sequences and nrITS were obtained for 42 taxa of *Cyrtanthus* (Table 1). Many of the unsampled species belong to two groups of closely allied species (the *C. macowanii* group and *C. loddigesianus* group) that are often difficult to distinguish from each other, but representatives from each of these groups, (*C. epiphyticus*, *C. macowanii*, *C. suaveolens*) and (*C. helictus*, *C. loddigesianus*, *C. smithiae*), are included in our sample. *Amaryllis belladonna* (Amaryllideae) was designated as functional outgroup for both gene regions, but *Calostemma luteum* (Calostemmateae) and *Clivia nobilis* (Haemantheae) were included in the matrix to allow the generation of bootstrap support percentages for a monophyletic *Cyrtanthus*. Leaf samples were collected from living collections at the Kirstenbosch National Botanical Garden, South Africa, and from populations in the field and they were preserved in silica gel for later extraction.

2.2. DNA extraction and amplification and sequence alignment

Genomic DNA was extracted from 30 mg of silica gel dried leaf tissue using the FastDNA Kit (BIO 101 Inc., Carlsbad, CA) according to manufacturer's protocols with a FP 120 FastPrep cell disrupter (Savant Instruments Inc., Holbrook, NY). Samples were quantified with a GeneQuant pro RNA/DNA calculator (Amersham Pharmacia Biotech Inc., Piscataway, CA, USA).

2.2.1. ndhF

The plastid *ndhF* gene was amplified using the primers of Olmstead and Sweere (1994) and Graham et al. (1998). The gene was amplified and sequenced as described by Pires and Sytsma (2002), but with 4% DMSO added to the 50 µl reaction mix.

2.2.2. ITS

Amplification and sequencing of the ribosomal DNA ITS1/5.8S/ITS2 region were accomplished using flanking primers (18S, 26S) AB101 and AB102 (Douzery et al., 1999), and the original White et al. (1990) internal primers ITS2 and 3 to amplify the spacers along with the intervening 5.8S gene as described by Meerow et al. (2000).

Amplified products were purified with an Exonuclease I and Shrimp Alkaline Phosphatase treatment. Cycle sequencing reactions were performed directly on purified PCR products using standard dideoxy cycle protocols for sequencing with dye terminators on either an ABI 3100 or ABI 3730 automated sequencer (according to the manufacturer's protocols; Applied Biosystems, Foster City, California, USA).

Both the ITS and *ndhF* sequences were readily aligned manually and unambiguously using Sequencher™ 4.8 (Gene Codes, Ann Arbor, MI, USA).

2.3. Phylogenetic analyses

The *ndhF* and ITS matrices were analyzed separately and in combination using parsimony with PAUP∗ v. 4.0b10 (Swofford, 2002), and with two model-based approaches, maximum likelihood (ML), utilizing TreeFinder (Jobb, 2008) and, for the combined analysis only, Bayesian analysis (BA), with MrBayes v. 3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). Best fit nucleotide substitution model was determined for each gene region with KAKUSAN v.3 (Tanabe, 2007), which also generates input files for these two programs. Best fit models were evaluated using the corrected Akaike Information Criterion (AICc; Akaike, 1973; Shono, 2000) for ML and the Bayesian Information Criterion (BIC) with significance determined by Chi-square analysis.

Parsimony tree searches were heuristic, conducted under the Fitch (equal) weights (Fitch, 1971) criterion with 2000 rounds of random addition sequence, saving no more than 20 minimum length trees per search for swapping using tree branch reconnection (TBR). Tree branches were collapsed if the