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THE NEVER-ENDING STORY: MULTIGENE APPROACHES TO THE PHYLOGENY OF AMARYLLIDACEAE

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ABSTRACT

Results to date with various plastid genes confirmed the monophyly of the Amaryllidaceae s.s. as a whole, strongly supported the mostly African tribe Amaryllideae as sister to the rest of the family, and resolved geographically-based monophyletic groups, but failed to resolve the relationships among several basal lineages in the family (the African Haemantheae and Cyrtantheae, the Australasian Calostemmataceae, and the American and European sister clades). We present analysis of plastid ndhF sequences that fully resolved the major clades of the family. The baccate-fruited Haemantheae and Calostemmataceae are sister tribes, and the African endemic Cyrtantheae is sister to them both. This clade is sister to an American/Eurasian clade. We also present preliminary nuclear ribosomal ITS sequence analysis of the Eurasian clade. Lycorideae are basal in the group and begin a grade that continues with *Himonia*, then *Pancreatum*, then *Lepidota*. The genera *Galanthus*, *Narcissus*, and *Sternbergia* are resolved as monophyletic with strong support. *Leucojum* is paraphyletic and recognition of *Acis* for the mostly autumn-flowering Mediterranean species is supported. Recent phylogenetic analyses of various tribes and genera of the family are reviewed. Above the family level, Agapanthoideae, Alliaceae, and Amaryllidaceae form a well-supported monophyletic group, but exact resolution of the relationships among the three subclades varies depending on the sequence matrix utilized. The Angiosperm Phylogeny Group II has advocated combining all three into a single family, Alliaceae. We discuss this decision, which has historical precedent, but recommend that Amaryllidaceae be conserved as the name for the family in such a treatment.

Key words: Amaryllidaceae, Asparagales, cladistics, DNA, monocotyledons, phylogeny.

INTRODUCTION

Amaryllidaceae are one of the few families of the higher Asparagales well defined by other than molecular characters, namely the combination of umbellate cymes, inferior ovaries, and unique alkaloid chemistry (Meerow and Snijman 1998), but morphological characters alone fail to adequately resolve phylogenetic relationships within the family (Meerow et al. 2000a). Some of these characters, such as inflorescence type, are likely synapomorphies for a deeper grouping of families (Agapanthaceae, Alliaceae) that could be subsumed in Amaryllidaceae (though this name would need to be conserved over Alliaceae). The four most recent infrageneric classifications of Amaryllidaceae are those of Traub (1963), Dahlgren et al. (1985), Müller-Doblies and Müller-Doblies (1996), and Meerow and Snijman (1998). Traub's scheme included Alliaceae, Hemerocallidaceae, and Ixioliricaceae as subfamilies, in part following Hutchinson (1934, 1959). Within this subfamily Amaryllidoideae, he erected two informal taxa, "infrfamilies" Amaryllidoideae and Pancratioideae, both of which were polyphyletic (Meerow 1995). Dahlgren et al. (1985) dispensed with any subfamilial classification above the level of tribe, recognizing eight, and treated as Amaryllidaceae only those genera in Traub's Amaryllidoideae. Stenomessaceae and Eustephiceae were combined. Meerow (1995) resurrected Eustephiceae from Stenomessaceae and suggested that two new tribes might need to be recognized, Calostemmataceae and Hymenocallidaceae. Müller-Doblies and Müller-Doblies (1996) recognized ten tribes (among them Calostemmataceae) and 19 subtribes, many of them monogeneric; Meerow and Snijman (1998) recognized 13 tribes, with two subtribes only in one of them. Discussion of character evolution within the family can be found in Meerow (1995), Meerow and Snijman (1998), and Meerow et al. (1999).

The precise relationship of Amaryllidaceae to other Asparagales remained elusive until Fay and Chase (1996) used molecular data to argue that Agapanthaceae, Alliaceae, and Amaryllidaceae form a monophyletic group (also evident in Chase et al. 1995) and that together they are related most closely to Hyacinthaceae s.s. and the resurrected family Themidaceae (the former tribe Brodiaeae of Alliaceae). They recircumscribed Amaryllidaceae to include *Agapanthus* L'Hé., previously included in Alliaceae, as subfamily Agapanthoideae. This recircumscription was based on phylogenetic analysis of plastid rbcL sequence data, with only four genera of Amaryllidaceae s.s. included in the analysis. All the epigynous genera were treated as Amaryllidoideae. Subsequent analyses of multiple DNA sequences from both the chloroplast and nuclear genomes have shown quite strongly that *Agapanthus*, Amaryllidaceae, and Alliaceae represent a distinct lineage within the monocot order Asparagales (Meerow et al. 1999; Fay et al. 2000), but the exact relationships among the three groups have been difficult to resolve with finality (Graham et al. 2006).

Meerow et al. (1999) presented cladistic analyses of plastid DNA sequences rbcL and trnL-F alone and in combi-
nation for 51 genera of Amaryllidaceae and 31 genera of related asparagalean families. The combined analysis was the most highly resolved of the three and provided good support for the monophyly of Amaryllidaceae and indicated Agapanthaceae as its sister family (though bootstrap support for this relationship was still weak at 60%). Alliaceae were in turn sister to the Amaryllidaceae/Agapanthaceae clade. In Fay et al.’s (2000) analysis of Asparagales using four chloroplast DNA regions, Alliaceae were resolved as sister to Amaryllidaceae, with Agapanthus sister to them both. Again, bootstrap support was weak at best. Based on these data, it would be possible to argue for recognizing Amaryllidaceae in a modified Hutchinsonian (1934) sense, i.e., with three subfamilies, Agapanthoideae, Allioidae, and Amaryllidoideae. Meerow et al. (1999) opted to recognize a monotypic Agapanthaceae. The latest Angiosperm Phylogeny Group (AGP II 2003) recommends treating all three as a single family, Alliaceae (which currently has nomenclatural priority), but also optionally suggest recognition of “Amaryllidaceae s.s.” using their “bracketing” system.

Based on the cladistic relationships of chloroplast DNA sequences (Meerow et al. 1999) the family originated in Africa and infrafamilial relationships are resolved along biogeographic lines. Tribe Amaryllideae, entirely southern African with the exception of pantropical Crinum L., were sister to the rest of Amaryllidaceae with very high bootstrap support. The remaining two African tribes of the family, Haemantheae (including Gethyllideae) and Cyrtantheae, were well supported, but their position relative to the Australasian Calostemmateae and a large clade comprising the Eurasian and American genera, was not clear. Most surprising, the Eurasian and American elements of the family were each monophyletic sister clades. Ito et al. (1999) resolved a very similar topology for a more limited sampling of Amaryllidaceae and related asparagoids using plastid matK sequences, but Agapanthus was sister to a diverse clade of Agavaceae, Anthericaceae, Funkiaceae, and Hyacinthaceae in their trees, the former three families represented by a single species each. There was no bootstrap support for this position of Agapanthus in their analyses.

The relationships of the endemic American genera were well resolved using the spacer regions of nuclear ribosomal DNA (Meerow et al. 2000b). Seventy-seven species of the monophyletic American Amaryllidaceae were analyzed using Pancratium L. as the outgroup. The American genera of the family form two major clades. The first, or “hippeastrid” clade, are diploid (n = 11), primarily the extra-American element of the family (though several of the genera do have American representatives), comprising the genera treated as the tribe Hippeastrae in most recent classifications (Dahlgren et al. 1985; Müller-Doblies and Müller-Doblies 1996; Meerow and Snijman 1998). The second clade constitutes the tetraploid-derived (n = 23) Andean-centered tribes. In addition, the Andean clade is characterized by three consistent deletions, two in the ITS1 and one in the ITS2 regions. Several genera within the hippeastrid clade resolve as polyphyletic (Rhodophiala C. Presl., Zephyranthes Herb.) and the possibility of reticulate evolution (i.e., early hybridization) in these lineages was hypothesized (Meerow et al. 2000b). A petiolate-leaved Andean subclade, containing elements of both Eucharideae and Stenomessaceae, was resolved with a bootstrap = 93%. In both of the major American clades, there is a small tribe that is sister to the rest of the clade, Eustephieae in the Andean group, and Griffineae in the hippeastrid clade. These two small tribes may represent either ancestral or merely very isolated elements of their respective clades.

To date, the relationships of the remaining endemic African tribal clades of the family after Amaryllideae branches remain unresolved. In the intervening years since Monocots II in Sydney (Wilson and Morrison 2000), we have been working on several fronts. A number of generic and tribal phylogenetic analyses have been completed (Meerow and Snijman 2001; Meerow et al. 2002, 2003; Meerow and Clayton 2004). In this paper we review the progress made on amaryllid phylogeny since Monocots II and present the first results of phylogenetic analyses across the entire family using plastid ndhF sequences, as well as preliminary analyses of the Eurasian clade of the family using ITS.

MATERIALS AND METHODS

Sampling

Genomic DNA was extracted from silica gel dried leaf tissue of the taxa listed in Table 1 as described by Meerow et al. (2000b). GenBank accession numbers are listed in Table 1.

DNA Extraction, Amplification, and Sequencing Protocols

Amplification of the ribosomal DNA ITS1/5.8S/ITS2 region was accomplished using flanking primers (in the 18S and 26S loci) AB101 and AB102 (Douzery et al. 1999) and the original White et al. (1990) internal primers ITS2 and ITS3 were used to amplify the spacers along with the intervening 5.8S gene as described by Meerow et al. (2000b). The plastid ndhF gene was amplified and sequenced using the eight primers of Olmstead and Sweere (1994). All polymerase chain reaction (PCR) amplifications were performed on a Gene Amp® PCR System 9700 (Perkin-Elmer Applied Biosystems, Foster City, California, USA).

Amplified products were purified using QIAquick (QIAGEN, Inc., Valencia, California, USA) columns, following manufacturer’s protocols. Cycle sequencing reactions were performed directly on purified PCR products on the ABI 3700, using standard dideoxy cycle protocols for sequencing with dye terminators on an ABI 3100 automated sequencer (according to the manufacturer’s protocols; Applied Biosystems).

Sequence Alignment

The ITS sequences of the Eurasian clade were aligned using CLUSTAL_X, applying various combinations of gap opening and extension penalties. The resulting alignments were then imported into Sequencher vers. 4.1 (Gene Codes Corp., Ann Arbor, Michigan, USA) for further manual editing. As there is a significant amount of sequence divergence among the major lineages in the Eurasian clade, the alignment used for the analysis presented here should be considered very preliminary. The ndhF sequences across the entire family were readily aligned using Sequencher alone.
Analyses

Aligned matrices were analyzed using the parsimony algorithm of PAUP* vers. 40b10 for Macintosh (Swofford 2001), with the MULPARS option invoked. Tree branches were retained only if unambiguous support was available (i.e., branches were collapsed if the minimum length = 0). Gaps were coded as missing characters. For the ndhF matrix, a branch-and-bound (Hendy and Penny 1982) search was conducted under the Fitch (equal) weights (Fitch 1971) criterion with a simple addition sequence. A heuristic search with 5000 random addition replications was conducted for the Eurasian clade ITS analysis, with tree-bisection-reconnection (TBR) branch swapping (saving no more than 100 trees from each replication). Node confidence was tested through 5000 replications of bootstrap analysis (Felsenstein 1985). Agapanthus praecox Willd. was used as the outgroup
for the ndhF analyses across the family, while Cyrtanthus herrei and Worsleya rayneri were used as outgroups in the ITS analyses of the Eurasian clade. Alignments and trees shown here are to be considered preliminary and part of larger works in progress, thus they have not been deposited in TreeBASE at this time.

RESULTS

Plastid ndhF Sequence Phylogeny of Amaryllidaceae

Of the 2098 total characters in our alignment, 103 were potentially parsimony informative. A branch-and-bound search found a single tree (Fig. 1) of length = 361 steps, consistency index (CI) = 0.86, and retention index (RI) = 0.82. The tribe Amaryllideae is the first terminally resolved branch of the tree with bootstrap support of 100%. Cyrtanthae are resolved as sister to a subclade of Haemanttheae (the baccate-fruited African Amaryllidaceae) and Calostemmataceae (the Australasian endemic clade of the family). There is <50% bootstrap support for the resolution of Cyrtanthus W. Aiton. The Haemanttheae/Calostemmataceae sister relationship have weak support (64%). This clade is in turn sister to the Eurasian/American clade, the latter with 100% bootstrap support. The American clade and the Eurasian clades have bootstrap support values of 100% and 99%, respectively. Lycorideae (from central and eastern Asia), repre-
Fig. 2.—Single most-parsimonious tree found by heuristic search of nuclear ribosomal DNA spacer sequence matrix for representative species of the Eurasian clade of Amaryllidaceae with *Cyrtanthus herrei* and *Worsleya rayneri* used as outgroups. Numbers below branches are bootstrap support percentages.

**ITS Sequence Phylogeny of the Eurasian Clade**

Of the 698 total characters in our current alignment, 339 were potentially parsimony informative. A single tree of length = 1140 was found (Fig. 2), with a CI of 0.60 and a RI of 0.73. While several genera are resolved as monophyletic with excellent bootstrap support (*Galanthus* L.: 100%, *Narcissus* L.: 100%, *Pancratium*: 89%, and *Sternbergia* L.: 86%), albeit with limited sampling. One species of *Leucojum* L. (*L. aestivum*) resolves as sister to *Galanthus* (83%) rather than as part of an otherwise monophyletic *Leucojum* (94% bootstrap).

The relationships among the terminal clades, however, mostly lack bootstrap support, except for the position of *Lycoris* Herb., which begins a basal grade in the Eurasian clade (bootstrap = 59%), followed by *Hannonia* Braun-Blanqu. & Maire (bootstrap = 63%), then *Pancratium*, and next *Lapiiedra* Lag. The remainder of the tree forms two sister clades.

Vagaria Herb. is sister to *Sternbergia* in one of them, and *Narcissus* is sister to *Galanthus/Leucojum* in the other.

**DISCUSSION**

**Overall Family Phylogeny**

The intrafamilial relationships of Amaryllidaceae resolved by ntdH sequences are completely congruent with the relationships supported by plastid *rbcL* and *trnL–F* sequences (Meerow et al. 1999), with additional benefit of increased bootstrap support for most of the clades, and the satisfying resolution of the basal polytomy that plagued the previous analyses. The African baccate-fruited Haemantheae and the Australasian Calostemmataceae are sister tribes, and *Cyrtanthaceae* are in tum sister to them both (though this latter relationship has <50% bootstrap support). *Cyrtanthus* retains the loculicidally dehiscent capsule and phytomelanous seed so common in the Eurasian/American clade. In so far as the globose, indehiscent capsule of Calostemmataceae resembles the unripe berry of many Haemantheae, this resolution has at least some morphological support. To this we might add the convergent evolution of a petiolate leaf with reticulate-like venation (*Scadoxus* Raf.) in Haemantheae and *Proiphys*...
Miuller-Doblies and Miuller-Doblies (1996) recognized four
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of subgen. 
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Charis
Carpolyza
and Boophone formed a grade at the base of their
trees (Fig. 3) and were recognized as the monotypic subtribes
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recognized: Crininae (which incorporates 
and Strumaria (which includes
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also recognized the polyphyly of
subtribes with little discussion and no phylogenetic analysis:
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sister to the rest of the tribe, with a monophyletic “Amaryllidae” (Brunsvigia, Hessea, Nerine, and Strumaria) nested
within an Amaryllis–Boophone–Crinum grade. The plastid
matK sequence analysis of Ito et al. (1999), who studied
only five taxa (Amaryllis, Brunsvigia, Crinum, Nerine, and Strumaria), also supported the basal position of Amaryllis.
Amaryllidaceae. Meerow and Snijman (2001) used a combination of nu-
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Fig. 3.—Phylogenetic tree derived from cladistic analyses of nuclear ribosomal DNA spacer sequences combined with morphological character matrix across the Amaryllidaceae tribe Amaryllideae (Meerow and Snijman 2001). Numbers above the lines are branch lengths. Numbers below the lines are bootstrap support percentages.
Fig. 4.—Phylogenetic tree derived from cladistic analyses of combined chloroplast trnL-F and nuclear ribosomal DNA spacer sequences across 43 species of *Crinum* and related genera (Meerow et al. 2003). Distribution of floral morphology and geographic position of the clades are shown.

...

Using three plastid DNA sequences, Meerow et al. (1999) confirmed the monophyly of Haemantheae, but indicated that Gethyllideae was embedded within the former tribe, and thus could not be recognized without rendering Haemantheae paraphyletic. The level of sampling and the number of phylogenetically informative DNA substitutions were insufficient to resolve the relationships within the tribe in that study beyond the well-supported sister relationship of Apodolirion and Gethyllis, which together terminated a successive grade beginning with Clivia, followed by Cryptostephanus, Scadoxus, and Haemanthus. However, bootstrap support for each branch in the grade was moderate to strong. Using plastid matK sequences, Ito et al. (1999), also resolved a monophyletic Haemantheae, though only three genera were sampled. Haemanthus and Scadoxus were sister taxa in their study, with 98% bootstrap support.

Using a combination of chloroplast and nuclear DNA sequences, Meerow and Clayton (2004) investigated the phylogeny of the Haemantheae across 19 species representing all genera of the tribe (Fig. 5). Two main clades were resolved, one comprising the monophyletic rhizomatous genera Clivia and Cryptostephanus, and a larger clade that unites Haemanthus and Scadoxus as sister genera to an Apodolirion/Gethyllis subclade. The second clade contains all of the genera that form true bulbs, though Scadoxus is polyphyletic for this character and has been misdiagnosed as being entirely rhizomatous (Friis and Nordal 1976). It is unclear whether bulbs form in Scadoxus only under certain environmental conditions or if bulb formation is limited to certain species. Biogeographic analysis rooted the tribe in eastern South Africa, with several subsequent dispersals to the winter rainfall western Cape region and tropical Africa. Chromosomal change from an ancestral $2n = 22$ (characteristic of Clivia) is associated with each main clade. Reduction in number has occurred in all but Cryptostephanus, which has $2n = 24$ chromosomes. Gouws (1949) noted the striking similarities between the karyotype of Clivia and Cryptostephanus. Cryptostephanus is the only member of the tribe with the ancestral state of phytomelan in the seed coat.

The sister relationship of Haemanthus and Scadoxus is well supported by the morphological synapomorphy of the brush-like inflorescence, facilitated by the reduction in perianth size (all species), and the dominance of the spathe bracts during anthesis; this occurs in at least some of the species of each genus (Friis and Nordal 1976; Nordal and Duncan 1984). Within Haemanthus, well-supported sister clades were resolved that corresponded to the eastern Cape (H. albilobus Jacq., H. humilis Jacq.) vs. the western Cape endemics (H. graniticus Snijman, H. pumilio Jacq.) (Snijman 1984). Scadoxus and Haemanthus have $2n = 18$ and 16 chromosomes, respectively (Vosa and Marchi 1980). Vosa and Marchi (1980) demonstrated that two small telocentric chromosomes in the karyotype of Scadoxus are homologous to one large, metacentric chromosome in the complement of Haemanthus and considered this to be an incidence of dispoist reduction.

One of four included Gethyllis species, G. lanuginosa Marloth, resolved as sister to Apodolirion in Meerow and Clayton’s (2004) analyses. Wilsenach (1965) found little variation among the karyotypes of representatives of both genera, all species of which so far investigated have $2n = 12$ chromosomes (Wilsenach 1965; Vosa 1986). Traub (1963) expressed doubt about maintaining Apodolirion and Gethyllis as distinct genera, an argument also taken up to some extent by Hilliard and Burtt (1973). They are differentiated by the capitulate stigma in Gethyllis (vs. trilobed in Apodolirion) and the often numerous stamens in Gethyllis (vs. six in Apodolirion). Gethyllis is most common in the winter rainfall region of South Africa, Apodolirion in the summer rainfall zone, but there are species of each in both climatic regions of the Cape. Clearly this question requires further investigation.

**Eurasian Clade**

Our tree is congruent with that of Lledo et al. (2004), in that the polyphyly of Leucojum is resolved with strong support. Lledo et al. (2004) resurrected the genus Acis Salisb. for the erstwhile Mediterranean Leucojum that resolve as sister to a Galanthus/Leucojum clade in their work as in ours (Fig. 2). However, their study focused on Galanthus and Leucojum with only a few other members of the Eurasian clade used as outgroups. This being the case, a straightforward comparison of our trees outside of the resolution of these two genera is not tenable. However, they also included a family-wide analysis combining the matK sequences of Ito et al. (1999) and the rbcL and trnL–F sequence data of Meerow et al. (1999). In those trees, Lycoris also resolves as sister to the rest of the Eurasian genera (bootstrap = 70%). Lapiendedra and Vagaria are sister genera, however (bootstrap = 70%), in turn sister to Galanthaceae (Galanthus, Acis, and Leucojum) in that combined plastid gene tree. ITS sequences for this group are plagued by both paralogy and significant divergence among the major groups, thus there is substantial room for error in constructing a larger alignment among the more easily aligned terminal (generic) clades. At present, the more limited sampling of this clade for ndhF (Fig. 1) is not congruent with the ITS phylogeny. We are developing a more extensive ndhF sequence matrix for the Eurasian group that we believe will provide well-supported resolution of these internal nodes.

**The Future of Amaryllidaceae**

It now appears that a well-resolved phylogeny of Amaryllidaceae is within our grasp. Similarly, the intimate relationship among Amaryllidaceae, Alliaceae, and Agapanthus appears unassailable at this point in time (see Graham et al. 2006). We are continuing our sampling with ndhF and ultimately will combine the preexisting plastid matrices with it, along with the 60-character matrix developed previously (Meerow et al. 2000a). As best as can be attempted without a fossil record, we would also like to try and date the significant divergence events in the evolutionary history of the

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Fig. 5.—Phylogenetic tree derived from cladistic analyses of combined chloroplast trnL-F and nuclear ribosomal DNA spacer sequences across 19 species of Amaryllidaceae tribe Haemantheae, with Amaryllis belladonna L. used as the outgroup (Meerow and Clayton 2004). Numbers above branches are branch lengths. Numbers below branches are bootstrap support.

We are also still attempting to understand the curious pattern of polyphyly resolved by ITS within certain genera of the American hippeastroids (Meerow et al. 2000b) and are currently testing various plastid spacer regions in the hopes that one or more will allow us to corroborate or challenge these results.

AGP II (2003) advocates recognizing all Agapanthaceae, Alliaceae, and Amaryllidaceae as three subtaxa of a single family, citing Alliaceae Batsch (1786) as the name of earliest priority. They do, however, leave open the option for recognizing three families. The former would essentially reestablish the family concept of Hutchinson (1934, 1959) with a few modifications. However, we believe that nomenclatural stability would be better served by conserving the name


