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SYSTEMATICS OF XANTHORRHOEACEAE SENSU LATO, WITH AN EMPHASIS ON *BULBINE*

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ABSTRACT

We provide here results of a combined analysis of plastid genes *rbc*L, *mat*K, and *ndh*F for Xanthorrhoeaceae s.l., the Asphodelaceae/Xanthorrhoeaceae/Hemerocallidaceae clade, which are well supported by the DNA data. *Xanthorrhoea* (often treated as the sole member of Xanthorrhoeaceae) is sister to the hemerocallid clade (former Hemerocallidaceae); and the asphodelid clade (formerly Asphodelaceae) is sister to them both. For additional species of *Bulbine* and *Jodrellia* (both Asphodeloideae), we also collected *rps*16 intron and ITS nuclear ribosomal DNA sequences to better assess their relationships. *Bulbine,* with *Jodrellia,* embedded are sister to the collective genera of subfamily Alooideae in which all species are characterized by strongly bimodal and nearly identical karyotypes, whereas that of *Bulbine* is much more variable. Cytological studies have previously shown *Bulbine* to possess a range of karyotypes from graduated to clearly bimodal (although never exactly like the aloid genera) and point toward a lower level of bimodality in the Australian members, all of which are autotetraploid, than in the African members, all of which are diploid. Therefore, there have been two events of particular interest within *Bulbine,* a change in ploidy and a long-range dispersal event.

Key words: Asparagales, Asphodelaceae, bimodal karyotype, *Bulbine,* Hemerocallidaceae, Xanthorrhoeaceae.

INTRODUCTION

Based on morphological and micromorphological characters, Dahlgren et al. (1985), following the concepts of Huber (1969), considered Asparagales as the largest of the five orders of the superorder Lilianae (they used Liliiflorae). Chase et al. (1995*a*) undertook the first extensively sampled molecular-based analysis to examine their circumscription. This analysis led to the recircumscription of Asparagales to include Orchidaceae (including the former Apostasiaceae and Cypripediaceae) and Iridaceae (including the former Geosiridaceae) and Dasypogonaceae s.l., Hanguanaceae, Luzuriagaceae p.p., with Philesiaceae being excluded. Since several morphological characters are shared with some Liliales and "lower" asparagoids (Rudall et al. 2000), the boundaries between Asparagales and Liliales can be difficult to define. Chase et al. (1995*b*) combined molecular and morphological data and found the same broad set of relationships, although the lilioid monocots were monophyletic, whereas in all other analyses they have been paraphyletic (Chase et al. 2006). Within Asparagales there is a well-supported lower asparagoid grade, characterized predominantly by simultaneous microsporogenesis, and a higher asparagoid clade in which all members exhibit successive microsporogenesis (Rudall et al. 1997). The Fay et al. (2000) analysis of a combined *rbc*L, *atp*B, *trn*L intron, and *trn*L–F intergenic spacer further resolved the phylogenetic patterns within Asparagales. Unless otherwise stated, all references to Xanthorrhoeaceae in this paper are to the larger unit circumscribed by the Angiosperm Phylogeny Group II (APG II 2003), which includes Asphodelaceae and Hemerocallidaceae, as well as Xanthorrhoeaceae s.s. Although there are no obvious morphological synapomorphies for Xanthorrhoeaceae s.l., some of them (Asphodelaceae) have been distinguished from Anthericaceae, with which they are often confused, by the lack of steroidal saponins and the frequent possession of hirsute anthers and anthraquinones.

We have a particular interest in *Bulbine* Wolf, one of the genera of Xanthorrhoeaceae within the asphodelid clade, because of its heterogeneity of karyotypes. *Bulbine* is a genus of mostly succulent species with an unusual African/Australian distribution. There are approximately 53 species distributed in southern and eastern continental Africa, with only three species in Australia. An inflorescence may bear up to 50 acropetally opening flowers in a pattern that is thought to reduce the likelihood of self-pollination. Flowers are fragrant, insect pollinated, and herkogamous. All African species are diploid ($2n = 14$) with no polyploidy (Baijnath 1977), whereas all Australian species are thought to be autopolyploid (Watson 1983). The most comprehensive study of the genus was carried out by Baijnath (1977); he delimited species using morphological characters, in particular, type of perennating organs, presence of auriculate or semi-auriculate bracts, number of tepal nerves, nature of stigmatic papillae, and relative lengths of leaves to inflorescences. Based upon these characteristics, Baijnath divided *Bulbine* into two subgenera, *Bulbine* and *Tuberosa. Jodrellia* was described by Baijnath (1978); of the three species in the genus, two were originally described as *Bulbine.* Baijnath proposed that *Jodrellia* should be separated from *Bulbine* for two reasons: firstly, all *Bulbine* species possess yellow or orange tepals, whereas *Jodrellia* possesses white ones. Secondly, the inner tepals of *Jodrellia* are one- nerved and the outer tepals possess three to five nerves, whereas both the

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inner and outer tepals of all *Bulbine* species possess a single nerve. Baijnath named *Jodrellia* in honor of the Jodrell Laboratory at the Royal Botanic Gardens, Kew, and hence its phylogenetic position was of particular interest to us.

Before this investigation, no molecular data were available for assessing relationships within *Bulbine*. Baijnath (1977) focused on karyological, morphological, and micromorphological characters; Baijnath and Cutler (1993) focused on leaf surface architecture using scanning electron microscopy and light microscopy, and Watson (1983) based her study on cytotypes, karyotypes, and nuclear DNA content.

In this study some of the taxonomic ambiguities surrounding Asparagales were investigated using molecular techniques, in particular relationships within the Xanthorrhoeaceae clade and the systematics of *Bulbine* within Asphodelaceae. Although we are working toward a comprehensive phylogeny of Xanthorrhoeaceae (including Asphodelaceae), the analysis of *Bulbine* for cytological, karyological, and molecular markers should be treated as a separate study to that of Xanthorrhoeaceae s.l. We were particularly interested in *Bulbine* because of its disjunctive distribution, cytological variation, and potentially complicated relationship to *Jodrellia*. We include both studies here to provide preliminary summaries of progress to date.

MATERIALS AND METHODS

For the Xanthorrhoeaceae and *Bulbine* molecular work, genomic DNA was extracted from both fresh and silica-dried material. The extractions were carried out following the 2× CTAB protocol (Doyle and Doyle 1987), but using a $CsCl_2$/ethidium bromide density gradient (1.55 g/μL) for purification.

Plant Material

Voucher information and GenBank accession numbers for taxa used in these analyses will be presented in a more detailed paper to follow these proceedings.

Amplification and Sequencing

Amplification of *mat*K was carried out in 50 μL reactions, containing 45 μL ABgene green master mix (2.5 mM Mg), 1.5 μL bovine serum albumin (0.4%), 0.6 μL H_2O, 0.7 μL of each *mat*K primer, −19F (Molvray et al. 2000) and 2R (Johnson and Soltis 1994) and exactly 60 ng DNA template. The polymerase chain reaction (PCR) profile was as follows: premelt of 94°C for 3 min, followed by 28 cycles of 94°C for 1 min, 50°C for 1 min, 72°C for 2 min in the first cycle, but then increasing by 10 sec every cycle, followed by a final extension of 7 min at 72°C.

Amplification of *rbc*L was carried out in a Perkin-Elmer 9700 PCR machine (Perkin-Elmer Applied Biosystems, Wellesley, Massachusetts, USA) using 50 μL reactions, containing 45 μL ABgene green mastermix (2.5 mM Mg), 1.5 μL BSA (0.4%), and 1.3 μL H_2O. Each sequence was assembled from half pieces using 0.6 μL each of 1F/724R and 636F/1460R primers (Muasya et al. 1998). Approximately 39 ng of template DNA were added to each reaction. The PCR conditions were: premelt of 94°C for 2 min, then 28 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, extension at 72°C for 2 min, followed by a final extension of 7 min at 72°C.

Primers and *ndh*F PCR and sequencing protocols are described in Pires et al. (2006). Amplification of internal transcribed spacer (ITS) for *Bulbine* and outgroups was in 50 μL reactions, containing 45 μL ABgene red mastermix (1.5 mM Mg), 1 μL BSA (0.4%), 0.3 μL H_2O, 0.6 μL of 17SE and 26SE of Sun et al. (1994), 2.5 μL dimethyl sulfoxide (DMSO) and approximately 39 ng DNA template. DMSO was added to reduce the effects of secondary structure, allowing the primers to bind efficiently and thereby making the PCR product reflect the number of copies in the genome, most of which are functional (Hung et al. 1990). The PCR protocol was premelt of 94°C for 2 min, followed by 28 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 1 min, extension at 72°C for 3 min, followed by a final extension of 7 min at 72°C.

Amplification of the *trn*L intron and the *trn*L–F spacer was carried out in 50 μL reactions, containing 45 μL ABgene green mastermix (2.5 mM Mg), 1.5 μL BSA (0.4%), and 1.3 μL H_2O. Each sequence was constructed from half pieces using 0.6 μL each of c/d and e/f primers of Taberlet et al. (1991). Approximately 39 ng of template DNA were added to each reaction. PCR conditions were premelt of 94°C for 2 min, followed by 28 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, extension at 72°C for 2 min, followed by a final extension of 7 min at 72°C.

Amplification of *rps*16 intron was carried out in 50 μL reactions, containing 45 μL ABgene green mastermix (2.5 mM Mg), 1.5 μL BSA (0.4%), and 1.3 μL H_2O. Each sequence was constructed from half pieces using 0.6 μL of c/d and e/f primers of Taberlet et al. (1991). Approximately 35 ng of template DNA were added to each reaction. PCR conditions were premelt of 94°C for 2 min, followed by 28 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, extension at 72°C for 2 min, followed by a final extension of 7 min at 72°C.

All PCR products were purified using QIAquick columns according to the manufacturer's protocols (QIAGEN, Ltd., West Sussex, UK). Dideoxy cycle sequencing was then performed using the chain termination method and ABI Prism Big Dye vers. 3.1 reaction kit following the manufacturer's protocols (Applied Biosystems, Inc. [ABI], Warrington, Cheshire, UK). The products were run on an ABI 3100 Genetic Analyzer, also according to the manufacturer's protocols. Sequence editing and assembly of contigs was carried out using Sequence Navigator and AutoAssembler software programs (ABI). All sequences were aligned by eye following the guidelines of Kelchner (2000); all insertions/deletions (indels) in *mat*K and *ndh*F were in triplets. Indels were not scored separately and thus were not included in the analyses.

SEARCH STRATEGY

All analyses were carried out using a combination of parsimony and bootstrapping. A Fitch parsimony method (equal weights, unordered characters; Fitch 1971) was employed using the software program PAUP* vers. 4.0b2A (Swofford 2001). Replicates of random taxon additions (1000) were

performed using the subtree pruning and regrafting (SPR) tree swapping method, with MulTrees on, but holding only five trees at each step to reduce the time spent in swapping on large numbers of trees. Support for clades was evaluated by bootstrapping (Felsenstein 1985) using 1000 replicates with simple sequence addition, SPR swapping, and holding five trees at each step.

Analyses were conducted using a successive weighting (SW) strategy (Farris 1969). Weights were assigned to characters using the "reweight characters" option based on a rescaled consistency (RC) index with a base weight of 1. When the tree length remained unchanged in successive rounds, these trees were accepted as the SW trees. The bootstrap procedure was carried out with the unitary weights, not the SW weights.

We combined all plastid data into one data set for the *Bulbine* analysis as it is a uniparentally inherited, nonrecombining genome; however, numbers of variable and informative characters for each region are also presented separately here (Table 1). The *Bulbine* data set was small enough to be analyzed using a combination of "branch and bound" and bootstrapping (1000 replicates).

RESULTS

Xanthorrhoeaceae Analysis

Relative to the two outgroups, Xanthorrhoeaceae s.l. are well supported (bootstrap percentage, BP 99; Fig. 1). *Xanthorrhoea* Sm. is sister (BP 70) to the former hemerocallid clade with weak support, but the hemerocallid clade itself is well supported (BP 91). Within the hemerocallid clade, there are two weakly supported subclades (BP 60, 62); the first has *Pasithea* D. Don sister to a moderately supported clade (BP 86) of *Phormium* J. R. Forst. & G. Forst., *Agrostocrinum* F. Muell./*Geitonoplesium* R. Br. ex Hook. (BP 98), and (*Dianella* Lam. ex Juss. (*Herpolirion* Hook. f./*Thelionema* R. J. F. Hend., BP 80) (*Rhuacophila* Blume/*Stypandra* R. Br., BP 88) BP 87)). In the second subclade *Hemerocallis* L./*Simethis* Kunth (BP 99) is weakly supported as sister to a well-supported clade in the form of a ladder with the following as successive sisters to the rest: *Tricoryne* R. Br. (BP 93), *Corynotheca* F. Muell. ex Benth. (BP 86), *Caesia* R. Br. (BP 82), *Arnocrinum* Endl. & Lehm. ex Lehm. (BP 100), and *Hensmannia* W. Fitzg. (BP 100) sister to *Johnsonia* R. Br./*Stawellia* F. Muell. (BP 70).

The asphodelid section is also strongly supported (BP 100). Within it there are two strongly supported subclades (BP100): *Asphodeline* Rchb./*Asphodelus* L. are sister to the rest (BP 93). Within this second subclade are two other moderately supported clades (BP 87 and BP 77). One clade has the moderately supported *Bulbine*/*Jodrellia* (BP 85) as sister to the very strongly supported *Aloe* L./*Haworthia* Duval clade (BP 100), which corresponds to subfamily Alooideae. The other contains the poorly supported *Eremurus* M. Bieb./*Trachyandra* Kunth section (BP 75) as sister to the *Bulbinella* Kunth/*Kniphofia* Moench clade (BP 81).

Bulbine *Analysis*

Relative to the two *Aloe* outgroups, *Bulbine* is not monophyletic (Fig. 2); however, there is strong support (BP 100)

Table 1. PAUP* analysis statistics.

Analysis	Region	Characters	Invariable	Variable but parsimony uninformative	Potentially parsimony informative	% potentially parsimony informative	Consistency index	Retention index
Xanthorrhoeaceae	Combined (*mat*K, *rbc*L, *ndh*F)	5964	4402	790	772	12.9	0.69	0.70
	*mat*K	1839	1225	288	326	17.7		
	*rbc*L	1158	840	171	147	12.7		
	*ndh*F	2697	2067	331	299	11.1		
Bulbine	Combined (*mat*K, *trn*L exon, *trn*L–F spacer, *rps*16 intron)	4148	3539	289	320	7.7	0.81	0.81
	*mat*K	1831	1584	135	112	6.1		
	*trn*L exon	50	38	5	7	14.0		
	*trn*L–F spacer	1044	780	131	133	12.7		
	*rps*16 intron	1223	1137	18	68	6.1		
	ITS	917	610	146	146	15.9		

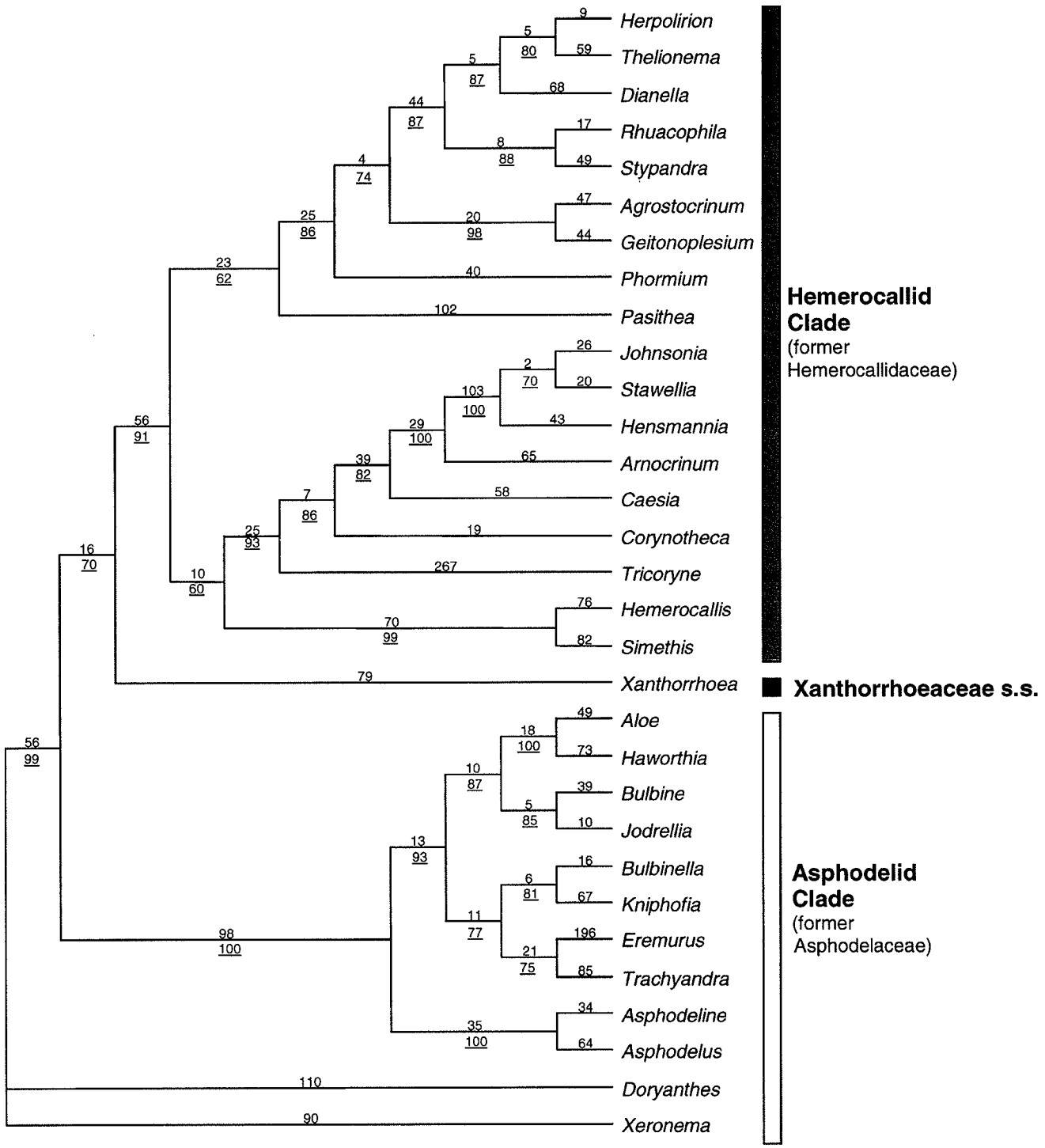

Fig. 1.—Combined tree produced by simultaneous analysis of the plastid genes *rbc*L, *mat*K, *ndh*F. Values above lines represent number of sequence changes. Underlined values are bootstrap percentages.

for a *Bulbine/Jodrellia* clade. Within *Bulbine/Jodrellia* there are two strongly supported subclades (BP 94 and BP 100) that correspond to subgen. *Bulbine* and *Tuberosa*, respectively. The subgen. *Tuberosa* clade has *Jodrellia* as sister to a strongly supported clade (BP 99) that contains two poorly supported subclades (BP 60 and 59). The first section contains a poorly supported clade (BP 71) that has *B. longifolia* Schinz as sister to the well-supported *B. praemorsa* (Jacq.) Roem. & Schult./*B.* species clade (BP 100). The second moderately supported section (BP 88) contains *B. bachmanniana* Schinz as sister to the strongly supported *B. alooides* (L.) Willd./*B. fallax* Poelln. clade. The clade corresponding to subgen. *Bulbine* has a moderately supported group (BP 85) containing the Australian taxa *B. glauca* (Raf.) E. M. Watson/*B. semibarbata* (R. Br.) Haw. as sister to the very strongly supported clade (BP 100) containing *B. abyssinicia*

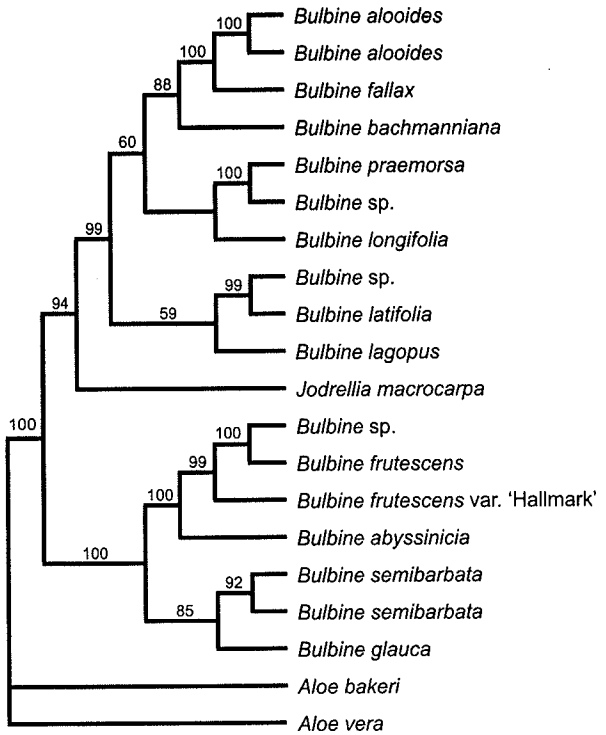

Fig. 2.—Combined *trn*L exon, *trn*L–F spacer, *rps*16, and *mat*K analysis. Single most-parsimonious tree obtained with successive weighting.

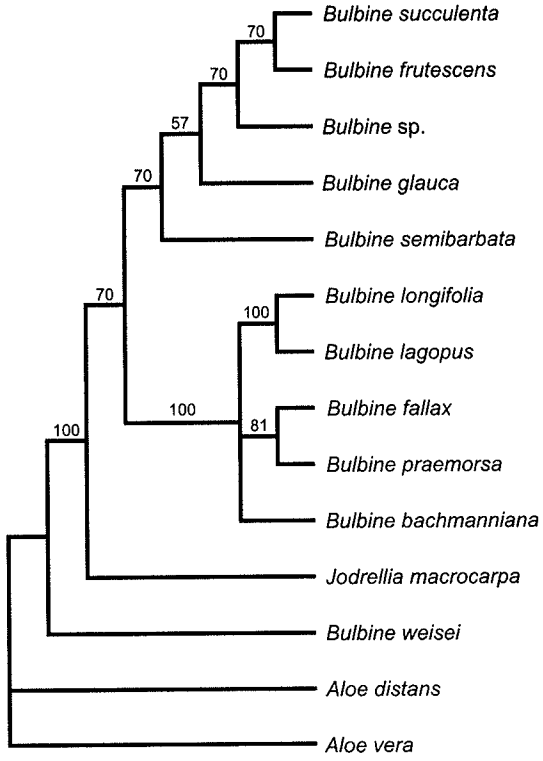

Fig. 3.—The strict ITS consensus of the three most-parsimonious trees obtained with successive weighting.

A. Rich. as sister to the strongly supported *B. frutescens* (L.) Willd./*B.* species group.

The strict consensus ITS tree (Fig. 3) shows two subclades within *Bulbine* that correspond to Baijnath's subgen. *Bulbine* and *Tuberosa*. *Jodrellia macrocarpa* Baijnath is embedded within *Bulbine* with 100% bootstrap support.

DISCUSSION

Within Asphodelaceae, all members of the monophyletic subfamily Alooideae are characterized by possession of aloin caps at their phloem poles (Beaumont et al. 1985), but this character has been little studied in the other genera of Xanthorrhoeaceae s.l. Aloin caps have also been reported in *Dianella* (Rudall et al. 2002). In this study, only two genera were used to represent Alooideae, *Aloe* and *Haworthia*. Alooideae are nested within Asphodeloideae, which also sporadically have aloin caps at phloem poles. They share some morphological characteristics with some members of Anthericaceae, such as six similarly shaped and arranged free stamens and tepals, but all exhibit simultaneous rather than successive microsporogenesis (Rudall et al. 1997). Within this subfamily there is a wide Old World distribution: western Asia, Europe, and southern and tropical Africa. *Bulbine* is one exception with its unusual African/Australian distribution, which is paralleled by the African/New Zealand distribution of *Bulbinella* and African/Australian distribution of *Caesia* (of the hemerocallid clade).

For information regarding the overall placement of Xanthorrhoeaceae see Pires et al. (2006). For Xanthorrhoeaceae, successive microsporogenesis (msg) is the norm with a reversal to simultaneous msg in *Xanthorrhoea* (Rudall et al. 1997). We now find that *Xanthorrhoea*, which had been placed by Fay (2000) as the weakly supported sister to Asphodelaceae, is with more data the weakly supported sister to the hemerocallid clade (BP 70). With additional outgroup data, Pires et al. (2006) found *Xanthorrhoea* as sister to the hemerocallids with BP 86. The placement of *Xanthorrhoea* with the hemerocallid and asphodelid clades in molecular analysis conflicts with its placement in a clade with Iridaceae in a morphological cladistic analysis (Rudall 2002), although the same analysis placed Asphodelaceae and Hemerocallidaceae together in an unresolved polytomy.

In the hemerocallid clade, the position of *Pasithea* is peculiar due to its distribution. *Pasithea* is a Chilean genus whereas other members of the clade have either a Eurasian (*Hemerocallis, Simethis*) or Australasian distribution (all the rest, except for *Caesia*, which is found in South Africa as well). Missing from this analysis is *Eccremis* Willd. ex Baker, which is also South American (it is similar in habit to *Phormium*). *Hemerocallis/Simethis* (BP 99) are weakly supported as sister to tribe Johnsonieae (of Anthericaceae sensu Dahlgren et al. 1985; all Australian, except for *Caesia*), whereas *Pasithea* is sister to the other largely Australasian clade (*Phormium* occurs in New Zealand, and *Dianella* is principally Australasian, but gets into tropical Asia at higher elevations). The pattern of relationships for *Pasithea* and unsampled *Eccremis* could be due to old Gondwanaland connections, but that of *Hemerocallis/Simethis* requires an explanation of an old long-distance dispersal or a much wider distribution for the group, with that of *Hemerocallis/Simethis* being relictual. The African/Eurasian primary distribution of the asphodelid clade (with an overlay of more recent long-

distance dispersal of *Bulbine* and *Bulbinella* to Australasia) only further complicates the biogeographical picture. A detailed molecular clock exercise is needed to provide dates on these critical nodes so that we have a better idea of when these events took place. Wikström et al. (2001), using the limited sampling of Asparagales in the combined analysis of Soltis et al. (2000), placed dates of 48–55 million years (depending on the method of optimizing branch lengths) for the node of *Xanthorrhoea* and *Bulbine,* which would correspond to the basal node within Xanthorrhoeaceae s.l. Older dates were obtained for this node in the Bremer and Janssen dating exercise (2006) for all monocots.

In the broader analysis, *Bulbine* (including *Jodrellia*) is sister to Alooideae. Both share characters such as succulent habit and CAM physiology (Watson and Dallwitz 1992 onwards), which could be seen as adaptations enabling them to live in similar arid environments. Within *Bulbine,* the results obtained generally validate the split into the tuberous and rhizomatous subgenera proposed by Baijnath. The only discrepancy is that of *B. lagopus* (Thunb.) N. E. Br., which in Baijnath's treatment was placed into subgen. *Bulbine,* but in the molecular study comes out in a strongly supported clade (BP 99) with the members of subgen. *Tuberosa.* The geographical evidence available indicates that subgen. *Bulbine* is a group of predominantly grassland species, whereas those in subgen. *Tuberosa* are inhabitants of drier areas, which fits with the rhizomatous and geophytic habits of these plants.

The pattern of evolution postulated by Baijnath (1977), in which *B. alooides* is proposed as a progenitor of the rest of the genus, is likely to be erroneous for two reasons. Firstly, *B. alooides* is an extant species and therefore by definition cannot be a progenitor of any other extant species in the genus. We think that most likely what Baijnath meant by this statement was that more than any other species *B. alooides* has the characteristics that he would have expected in the common ancestor of all extant species. This could be the case, although the deeply embedded and well-supported position of this species (Fig. 2) would seem to argue against even this interpretation, but without a formal cladistic analysis of morphology we cannot robustly address this hypothesis. On the basis of the tree, it is not particularly parsimonious to expect *B. alooides* to have retained many plesiomorphic characters. Secondly, he proposed that Australian members (*B. glauca* and *B. semibarbata*) arrived on the continent via two long-range dispersal events and so are distinct and temporally separated lineages. The low level of sequence divergence in these species does not support this hypothesis, but instead indicates that they are monophyletic and recently arrived in Australia.

A Gondwanic origin had been suggested for the split between the African and Australian *Bulbine* species (Baijnath 1977); however, for a split that took place 100 million years ago (Hallam 1973), the level of sequence divergence is too low. At some point there was a change in ploidy in *Bulbine* and a long-range dispersal event. At this stage it is still not possible to say in which order these happened, but since there are no tetraploid species in Africa they either left no relatives there or the change in ploidy occurred after the ancestral species arrived in Australia.

Whereas all African members investigated have $2n = 14$, chromosome numbers of $2n = 24$, 26, 48 and 72 have been reported for the Australian members (Watson 1987). Africa is clearly the most likely origin of *Bulbine,* with the basic chromosome number of $x = 7$ as ancestral. As there are only examples of $2n = 14$ from Africa, it is possible that *B. semibarbata* was formed as a result of "dysploid drop" in which there was a doubling of the African chromosome number to $2n = 28$, followed by a subsequent fusion of chromosomes in Australia.

Support for *Bulbine* and *Jodrellia* being in the same clade is high, which is backed up by both nuclear and plastid data sets (Fig. 2, 3). *Jodrellia* should be considered a synonym for *Bulbine*; only one combination in *Jodrellia* has never been made in *Bulbine.* The bimodal karyotype in *Bulbine* has evolved independently of that in the *Aloe* clade. All members of the latter group have exactly the same karyotype, which is not shared by any *Bulbine* species, whereas the species of *Bulbine* display a wide range of karyotypes, including some species that are more or less uniform in shape and size or exhibit a continuum of small to large chromosomes (Chase et al. 2000). The latter appears to be ancestral for the genus, as the more bimodal ones are not attached to the basal node of the tree. However, additional species need to be included before such conclusions can be made robustly. The type of bimodality found in *Gasteria* Duval/*Haworthia* also matches that found in *Aloe* with four large and three small chromosomes (Pires et al. 2006), indicating that this is the basic karyotype found in Alooideae, although the limited sampling in the asphodelid clade and the lack of a morphological matrix means that we can only talk in general terms about the patterns observed. The strict consensus ITS tree (Fig. 3) is provided to back up the findings from the plastid investigation. As can be seen, the same broad sets of relationships were indicated including the same clades (corresponding to Baijnath's subgen. *Bulbine* and *Tuberosa*) and most notably the position of *Jodrellia,* as with the combined plastid data set tree, was embedded within *Bulbine* with 100% bootstrap support. The data sets were not combined due to the incomplete sampling of the ITS tree.

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