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Molecular phylogenetics of Hypoxidaceae – Evidence from plastid DNA data and inferences on morphology and biogeography

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ABSTRACT

Phylogenetic relationships of the monocot family Hypoxidaceae (Asparagales), which occurs mainly in the Southern Hemisphere, were reconstructed using four plastid DNA regions (*rbcL*, *trnL* intron, *trnL-F* intergenic spacer, and *trnS-G* intergenic spacer) for 56 ingroup taxa including all currently accepted genera and seven species of the closely related families Asteliaceae, Blandfordiaceae, and Lanariaceae. Data were analyzed by applying parsimony, maximum likelihood and Bayesian methods. The intergenic spacer *trnS-G* – only rarely used in monocot research – contributed a substantial number of potentially parsimony informative characters. Hypoxidaceae consist of three well-supported major clades, but their interrelationships remain unresolved. Our data indicate that in the *Pauridia* clade one long-distance dispersal event occurred from southern Africa to Australia. Long-distance dispersal scenarios may also be likely for the current distribution of *Hypoxis*, which occurs on four continents. In the *Curculigo* clade, the present distribution of *Curculigo* s.s. on four continents could support a Gondwanan origin, but the level of divergence is too low for this hypothesis to be likely. The main clades correspond well with some floral characters, habit and palynological data, whereas chromosomal data exhibit plasticity and probably result from polyploidization and subsequent dysploidy and/or aneuploidy. Evolutionary flexibility is also suggested by the number of reported pollination syndromes: melittophily, myophily, sapromyophily, and cantharophily. Based on our phylogenetic results, we suggest cautious nomenclatural reorganization to generate monophyly at the generic level.

1. Introduction

The monocot family Hypoxidaceae occurs mostly in the Southern Hemisphere with only a few species in the Northern Hemisphere (Table 1). In temperate southern Africa, Hypoxidaceae are important elements of spring- and summer-flowering bulb floras, and in Southeast Asia they can be the dominant herbs in secondary tropical forests. The family consists of nine genera and ca. 155 species, of which the largest is *Hypoxis* with up to 90 species (Nordal, 1998). The plants are herbaceous and mostly 20 cm or less tall, although a few reach a meter or more, all with a tuberous or elongated rhizome or corm (Fig. 1). Flowers of Hypoxidaceae are usually less than 2 cm in diameter and yellow, white, pink, or rarely orange. They are mostly scentless and offer pollen as a reward. A genus endemic to the Seychelles, *Hypoxidia*, has large red-brown[1] fetid flowers (Fig. 1E). General flower structure follows the "typical" lily-like monocot pattern with three sepals, three petals, six stamens, and a trimerous gynoecium (Remizowa et al., 2010). However, two remarkable exceptions exist: *Pauridia* from South Africa has only three functional stamens with the three stamens of the outer staminal whorl reduced to staminodes that terminate in minutely papillate hooks, and *Curculigo racemosa* from Borneo can be polyandrous, often combined with a variable number of carpels (Thompson, 1978; Burtt, 2000; Rudall and Bateman, 2002; Kocyan, 2007). Hypoxidaceae are one of 12 families (Asteliaceae, Blandfordiaceae, Boryaceae, Doryanthaceae, Iridaceae, Ixioliriaceae, Lanariaceae, Orchidaceae, Tecophilaeaceae, Xanthorrhoeaceae s.l.,

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[1] For interpretation of color in Fig. 1, the reader is referred to the web version of this article.

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Table 1
Genera currently included in Hypoxidaceae R.Br. with their species numbers and geographic range.

Genera	No. of described species	Distribution
Curculigo Gaertn.[a]	±6	Africa, Asia, Australia, South-Central America, Seychelles
Empodium Salisb.	7	Southern Africa
Hypoxidia F. Friedmann	2	Seychelles
Hypoxis L.	±90	Africa, Asia, Australia, Americas
Molineria Colla	±10	Asia
Pauridia Harv.	2	Southern Africa
Rhodohypoxis Nel	6	Southern Africa
Saniella Hilliard & B.L.Burtt	2	Southern Africa
Spiloxene Salisb.	±30	Southern Africa

Information of species numbers gathered from the following sources: Nordal (1998), Snijman (2000), Snijman and Singh (2003), Singh (2006, 2007), and World Checklist of Selected Plant Families, 2010.

[a] Ravenna (2003) separated the genus *Heliacme*, which includes solely the Latin American *Heliacme scorzonerifolia* (formerly *Curculigo scorzonerifolia*), from *Curculigo* stating that *Heliacme* is different in having an unilocular ovary, dry capsulate fruit, non-sagittate anthers and a one-flowered inflorescence. Unfortunately, Ravenna did not illustrate his result with line drawings or photographs, which would allow a verification of these characters. The first author of the present article has seen herbarium sheets with individuals of *Curculigo scorzonerifolia* that have several flowered racemes. Also there are clearly sagittate anthers visible on line drawings of *Curculigo scorzonerifolia* in floristic literature. Literature is non constistent about dry or succulent fruits in *Curculigo*. J. Dutilh reports fruits that are not dry (pers. comm.). Whether an ovary is unilocular or 3-locular can only be decided by detailed morpholgical studies and so far we are unaware of any publication on this subject, given that the ovary is very small. However, the authors prefer to keep the *Heliacme* in *Curculigo* for this study.

and Xeronemataceae) that together constitute the 'lower asparagoid' grade of the order Asparagales. These asparagoid lilies are characterized by predominantly simultaneous microsporogenesis and an inferior ovary (Rudall et al., 1997). Within this grade, four families (Asteliaceae, Blandfordiaceae, Hypoxidaceae and Lanariaceae) form a well-supported clade (Chase et al., 2006). Boryaceae also have been found to belong to this clade (Graham et al., 2006; Pires et al., 2006). Monophyly of Hypoxidaceae, confirmed by cladistic analyses of *rbcL* DNA sequences (Rudall et al., 1998), is supported by several micromorphological characters. Synapomorphies that unite Hypoxidaceae are the presence of bulliform cells in leaves, successive microsporogenesis, and tenuinucellate ovules. Two other characteristic features, mucilage canals in a wide range of organs and branched trichomes on leaves, occur within the family, but these two are also shared with Asteliaceae.

Although hypotheses of the systematic position of the family have been consistent in molecular analyses, only a modest set of information has been gathered on the intrafamilial relationships of Hypoxidaceae. Rudall et al. (1998) included seven of the nine genera, but did not analyse multiple species from most of these (see below). Nordal (1998) proposed some clades based on geographical and basic morphological information. She suggested two primary groupings: (1) *Curculigo*, *Hypoxidia* and *Molineria* centered around the Indian Ocean and (2) *Empodium*, *Pauridia*, *Rhodohypoxis*, *Saniella*, *Spiloxene* and *Hypoxis* focused mainly in southern Africa; *Hypoxis* was regarded as sister to the southern African genera because of its much wider distribution (more or less cosmopolitan, except Europe). Earlier, Thompson (1978) separated the southern African genera still further, placing *Hypoxis* and *Rhodohypoxis* in one group and *Empodium*, *Spiloxene* and *Pauridia* in another.

According to Rudall et al. (1998; Fig. 2), who analyzed *rbcL* DNA sequences and morphological data, the family is divided into two major clades. In contrast with Nordal's (1998) proposal, *Molineria* (based on *Molineria capitulata,* but with the voucher referred to as a species of *Curculigo*), *Empodium* and *Hypoxidia* formed one clade. The second clade consisted of two subclades, of which the first contained *H. leptocarpa* (=*H. curtissii)* and *Rhodohypoxis*, and the second contained *Hypoxis glabella*, *Spiloxene* and *Pauridia*. This indicated that *Hypoxis* is polyphyletic, an unexpected finding; *H. leptocarpa* (=*H. curtissii)* occurs in North America, whereas *H. glabella* is from Australia. All other members of this clade (*Pauridia*, *Rhodohypoxis*, *Spiloxene*) are from southern Africa. Thus far, no *Hypoxis* species from Africa – the center of diversity for *Hypoxis* – have been analyzed in a phylogenetic context at the family level. All other genera in the Rudall et al. (1998) study were represented by only a single species.

Generic limits in Hypoxidaceae have always been problematic. *Hypoxis* has been variously treated by several authors (Baker, 1878; Nel, 1914b; Garside, 1936; Geerinck, 1969), whereas separation of *Pauridia* from *Spiloxene* has remained uncertain (Burtt, 2000). Ongoing taxonomic confusion also surrounds the identity of *Curculigo* and *Molineria*, and often the species of the latter have been treated as *Curculigo* (e.g. Geerinck, 1969, 1993). Henderson (1987) stated that differences exist in each organ, such as stem system, flowers, fruit and seeds, that warranted keeping the two genera distinct. This ambiguity was partly caused by the somewhat overlapping diagnostic characters for the two genera. Nel (1914a) distinguished them on the presence of beaked (*Curculigo*) or unbeaked (*Molineria*) fruits. However, the beak character is not consistent because several species placed in *Molineria* are clearly beaked (Fig. 1K and L). A more consistent but difficult to observe diagnostic character was proposed by Hilliard and Burtt (1978), who studied stamen morphology in detail and showed that the anthers in transverse section are asymmetrical in *Molineria* and symmetrical in *Curculigo*. However, observations by Kocyan and Endress (2001b) and Kocyan (2007) indicated that *Molineria* has asymmetrical as well as symmetrical anthers (*Molineria* was treated there as *Curculigo* for *C. capitulata* and *C. latifolia*). At present, the only clear morphological distinction between the two genera is the presence in *Curculigo* of prominently beaked seeds, in which the funicle has an expanded end and the seed surface is usually striately ornamented (Henderson, 1987; Wiland, 1997; Nordal, 1998). In contrast, the seeds of *Molineria* are unbeaked, generally smaller than *Curculigo* seeds, and the surface is striate or not with a subtle tesselate ornamentation (pers. obs., Kocyan). The overlapping distributions of the two genera have added to the confusion; *Curculigo* occurs throughout the tropics and subtropics, whereas *Molineria* occurs naturally only from southern Asia to northern Australia.

Hypoxidaceae have been proposed as a potential sister family of Orchidaceae (e.g. Garay, 1960, 1972; Hutchinson, 1973) based on morphological features. *Curculigo* and *Molineria* show some similarity in leaf characters with the two genera of Apostasioideae, which are sister to the rest of Orchidaceae: both genera are herbs with plicate palm-like leaves found in forests in Southeast Asia. Moreover, *Apostasia* and *Molineria* (=*Curculigo*) *capitulata* both have *Solanum*-type flowers with united stamens, most probably exhibiting a buzz-pollination syndrome (Kocyan and Endress, 2001a, b). A further similarity with orchids is found in the small hypoxid genus

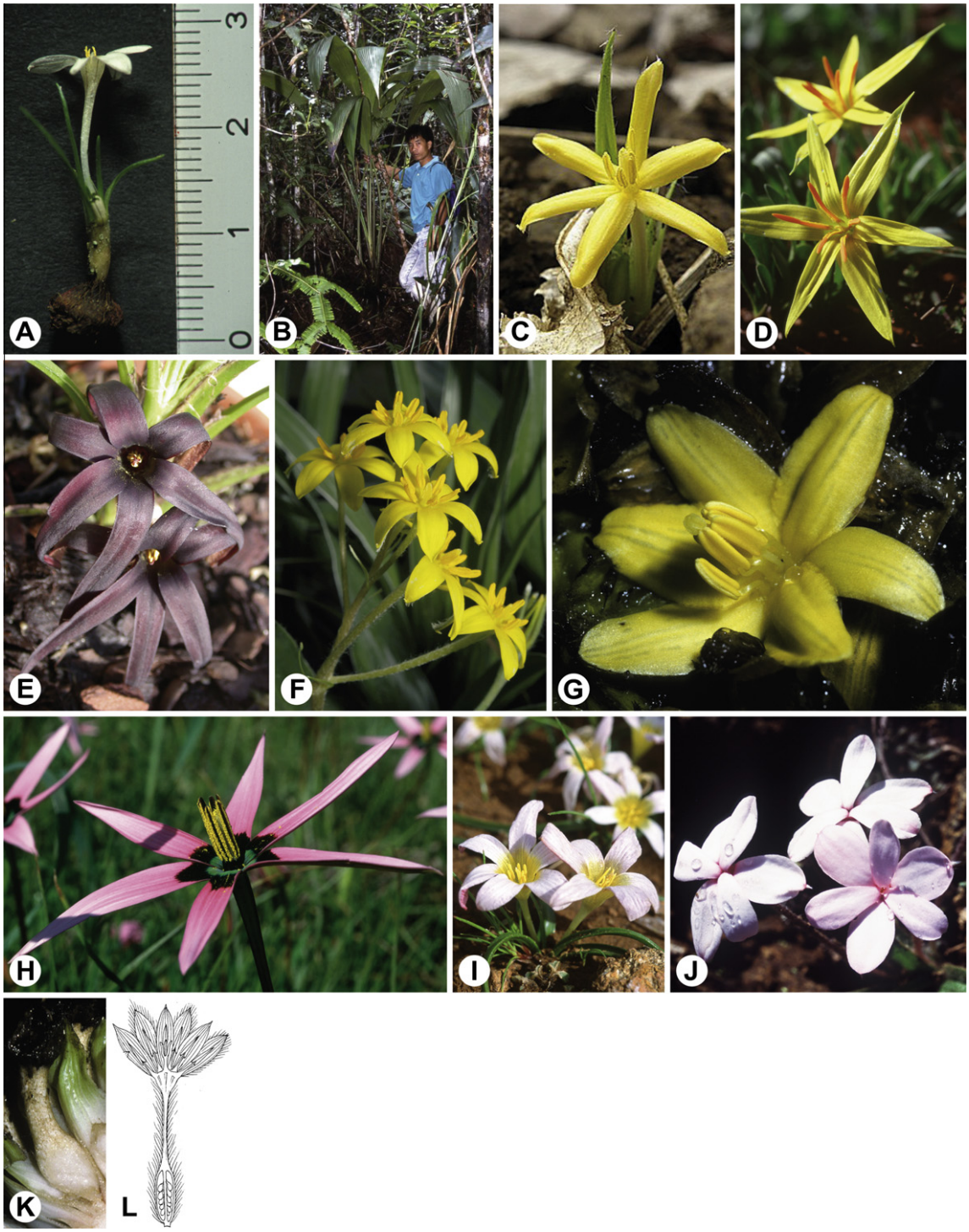

Fig. 1. Representatives of all hypoxid genera in flower (A, C–J). Habit size range within Hypoxidaceae comparing *Pauridia longituba* (A) and *Molineria latifolia* (B). Homoplasious evolution of floral rostra in *Molineria latifolia* (K) and *Curculigo orchioides* (L). (A) *Pauridia longituba*. (B, G, K) *Molineria latifolia*. (C and L) *Curculigo orchioides*. (D) *Empodium flexile*. (E) *Hypoxidia rhizophylla*. (F) *Hypoxis setosa*. (H) *Spiloxene capensis*. (I) *Saniella occidentalis*. (J) *Rhodohypoxis baurii*. Photos: A, B, F, G, K by A. Kocyan; C by R. Sachdev; D, H, I, J by C. Paterson-Jones; E by Ludwig Beenken; L from Wight R. 1853. Icones Plantarum Indiae Orientalis. T. 2043.

Pauridia from South Africa, which lacks the outer stamen whorl and has only three stamens from the inner stamen whorl (Rudall and Bateman, 2002). Although orchids possess either three stamens (*Neuwiedia*, Apostasioideae), two stamens (*Apostasia*, Apostasioideae; Cypripedioideae) or (in the vast majority of orchids), only one stamen, *Pauridia* is unlikely to be the closest extant sister taxon of Orchidaceae because the functional stamens of orchids correspond only partially with those of *Pauridia*. However, *Pauridia* exhibits characters that may still be important to understanding orchid floral evolution (Rudall and Bateman, 2002).

The economic importance of Hypoxidaceae lies mostly in their use as traditional medicines, especially in Africa where research has shown that *Hypoxis hemerocallidea* could have some benefit in the treatment of HIV and certain tumours (Albrecht et al., 1995; Drewes and Khan, 2004; Mills et al., 2005). *Curculigo orchioides* is used for a variety of diseases in traditional Indian

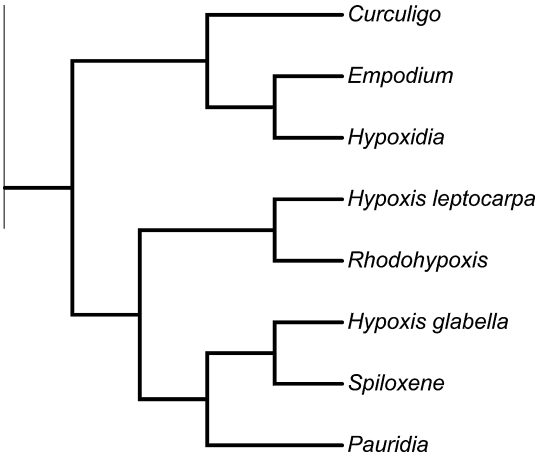

Fig. 2. Topology of Hypoxidaceae based on the combination of *rbcL* sequence and morphological data (redrawn after Rudall et al., 1998).

herbal treatments and was successfully tested for antidiabetic activity (Madhavan et al., 2007), and *H. aurea* is included in the Chinese pharmacopeia (Judd, 2000). Various indigenous peoples also harvest the leaves for their fibers. In southern Africa, *H. rigidula* and *H. hemerocallidea* are used for making rope (Watt and Breyer-Brandwijk, 1962), and in Borneo species of *Molineria* are harvested for weaving head straps and burden baskets and for their sweet, edible berries. *Molineria*, *Hypoxis* and *Rhodohypoxis* are occasionally used as ornamentals.

It is timely to conduct a new phylogenetic study of Hypoxidaceae including all genera with multiple species from each genus. For this purpose, we analyzed both existing and new sequences of the *rbcL* gene in combination with putatively more rapidly evolving plastid DNA regions. We included the *trnL-F* intron/spacer region and the *trnS-G* spacer (Taberlet et al., 1991; Hamilton, 1999). The *trnL-F* region has been used extensively in a vast number of phylogenetic studies. However, the molecular evolution of *trnL* intron and in particular the P8 part is only partly understood (Kocyan et al., 2008 and references therein). In *Aerides* (Vandeae; Orchidaceae) a detailed study of P8 lengths and secondary folding patterns revealed additional characters to distinguish between the three sections of that genus (Kocyan et al., 2008). The *trnS-G* region has been used in various phylogenetic studies in eudicots (e.g. Schönenberger and Conti, 2003) but it has not hitherto been exploited in monocots because Hiratsuka et al. (1989) found that in rice the *trnS-G* region is interrupted by an inversion preventing amplification. However, studies on the orchid genera *Renanthera* (Hofmann, Renner, Kocyan unpublished), *Liparis* (Tsutsumi et al., 2007) and *Satyrium* (van der Niet and Linder, 2008) revealed that this region can be amplified and is phylogenetically informative.

This paper aims to address the following topics: (1) phylogenetic relationships among the genera of Hypoxidaceae; (2) the biogeographic history relative to the age of the family; and (3) any conspicuous features of the DNA regions included here, in particular in the *trnL* intron.

2. Material and methods

2.1. Sampling

In total, we sampled 63 taxa, of which 56 were Hypoxidaceae (Table 2). Based on Chase et al. (2006), seven representatives of Asteliaceae, Blandfordiaceae and Lanariaceae were chosen as outgroup taxa.

2.2. DNA extraction, PCR and sequencing

DNA was extracted from silica gel-dried leaf material following the commercially available NucleoSpin extraction kit of Machery-Nagel (Düren, Germany) or E.Z.N.A. Plant DNA Kit of Omega Bio-Tek (Doraville, GA, USA) according to the manufacturers' protocols; for several species, DNA was obtained from well-preserved herbarium specimens.

The DNA regions were amplified with the standard polymerase chain reaction (PCR). The primers 1F and 1460R were used to amplify most of the *rbcL* gene (Olmstead et al., 1992; Fay et al., 1997); the primers c and f of Taberlet et al. (1991) were used to amplify the *trnL* (UAA)5′ exon to the *trnF* (GAA) gene (the *trnL-F* region); the S and G primers to amplify the spacer region between *trnS-trnG* (Hamilton, 1999). The PCR protocol used to amplify *rbcL*, *trnL-F* and *trnS-G* was as follows: initial denaturation at 94 °C for 5 min., 32 cycles of 1 min at 95 °C denaturation, 1 min annealing (48 °C for *rbcL*, 55 °C for *trnL-F* and *trnSG*), 1 min 40 s elongation at 72 °C, followed by a final elongation period of 7 min. at 72 °C. In a few cases, the *trnL-F* region had to be amplified in two non-overlapping parts using the primers d and e (Taberlet et al., 1991). For some *trnS-G* PCR reactions and some of the herbarium DNAs the more reactive KOD Hot Start DNA Polymerase by NOVAGEN was used successfully.

PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Basel, Switzerland), the Wizard SV Gel and PCR Clean-up system (Promega, Wisconsin, USA), GenElute PCR Clean-Up Kit (Sigma–Aldrich, Buchs, Switzerland) or were treated enzymatically prior to cycle sequencing reactions. Cycle sequencing reactions used the same primers as for PCR with the Big Dye Terminator kit v3.1 (Applied Biosystems, Warrington, UK) according to the manufacturer's protocol except that we used 1/16 of the recommended volumes of Big Dye and compensated for the reduction in salt concentration; additional primers were used for sequencing: 600F and 724R (monocot) for *rbcL* (Asmussen and Chase, 2001; Kocyan et al., 2007), and d and e for *trnL-F* (Taberlet et al., 1991). Occasionally, the primers c2 and F3 were used instead of the normal primers c and f for PCR and sequencing reactions for the *trnL* region (Bellstedt et al., 2001; Meimberg et al., 2003). After cycle sequencing, products were cleaned by Sephadex G-50 Superfine gel filtration (Amersham) on MultiScreen TM-HV membrane plates (Millipore, Bedford, USA) according to the manufacturer's protocol or with the Big Dye XTerminator Purification Kit of Applied Biosystems, Inc. (ABI). Fragments were separated on ABI 3100, ABI 3130, ABI 3130xl, ABI 3100 Avant or ABI 3730 capillary sequencers.

2.3. Sequence editing, alignment and the P8 region

Sequence assembly and editing were conducted using the software Sequencher (vs. 4.7; Gene Codes, Ann Arbor, Michigan, USA). Sequence contigs were generally bidirectional. All sequences were deposited in GenBank; accession numbers are provided in Table 2. Sequences were aligned by eye using MacClade 4.08 for OS X (Maddison and Maddison, 2005) as computerized alignments have been found to be of only limited help for phylogeneticists (Morrison, 2009). Due to ambiguous alignments, we excluded from the *trnL* and the *trnS-G* regions several nucleotide regions (401 of 1142 and 503 of 1494 aligned positions, respectively). The datamatrix is available from the first author upon request.

Within the *trnL* intron, a highly variable region containing a long TA repeat is often conspicuous. This hypervariable region is part of the P8 stem-loop segment of the intron. Earlier studies revealed that the length differences between clades of *Aerides* (Orchidaceae) may contain some potentially useful characters (Kocyan et al., 2008). In order to test the taxonomic value of P8 length within Hypoxidaceae, we identified the borders of the P8

Table 2
Species and gene regions sequenced for this study, voucher information, geographic origin, geographic range and GenBank accession numbers. Asterisks indicate the types of the genera.

Species	DNA source / Voucher	Geographic origin of the sequenced material	Geographic range	*rbc*L gene	*trn*L intron	*trnL-F* spacer	trnGS spacer
Hypoxidaceae							
Curculigo erecta Lauterb.	cult. BG Leiden 932789	Sarawak	Borneo	HM459539	HM459485	HM459485	HM459437
Curculigo finlaysoniana (Baker) Wall. ex Hook.f.	C.D.K. Cook, E.M. Rix & R.J. Schneller No. 23 (Z/ZT)	Kerala/India	Southern India, Sri Lanka	HM459540	HM459486	HM459486	–
∗*Curculigo orchioides* Gaertn.	(1) coll. M.K. Janarthanam, s.n., Goa University Campus	(1) Goa/India	Tropical and subtropical Asia and West Pacific	(1) HM459541	(1) HM459487	(1) HM459487	(2) HM459438
	(2) TCMK 696 (K)	(2) Guizhou Prov., China					
Curculigo pilosa ssp. *major* (Baker) Wiland	R. Ehrich, No. 266 (B)	Mali	Tropical Africa	HM459542	HM459488	HM459488	HM459439
Curculigo racemosa Ridl.	cult. BG Leiden 970647	Sarawak	Borneo	HM459543	HM459489	HM459489	HM459440
Curculigo scorzonerifolia (Lam.) Baker	A.R.A. Görts et al. nr. 449 (L ex U)	Venezuela	South Mexico to tropical America	HM459544	HM459490	HM459490	HM459441
Curculigo seychellensis Bojer ex Baker	(1) leg. C. Küffer, s.n., 28. 11. 2002	Seychelles	Seychelles	(1) HM459545	(1) HM459491	(1) HM459491	(1) HM459442
	(2) cult. BG Bonn			(2) HM459546	(2) HM459492	(2) HM459492	(2) HM459443
Curculigo sinensis S.C.Chen	cult. Kunming Botanical Garden, s.n.	–	China	HM459547	HM459493	–	HM459444
Empodium elongatum (Nel) B.L.Burtt	Snijman 1908 (NBG)	N Cape, Hanover	South Africa	HM459553	HM459495	HM459495	HM459445
Empodium flexile (Nel) M.F.Thomps. ex Snijman	Snijman 1706 (NBG)	N Cape, Nieuwoudtville	South Africa	HM459554	HM459494	HM459494	HM459446
∗*Empodium plicatum* (Thunb.) Garside	cult. BG Munich, s.n. (M)	–	South Africa	HM459555	HM459496	HM459496	HM459447
Empodium veratrifolium (Willd.) M.F.Thomps.	Kocyan AK990309/1/03 (Z/ZT)	–	South Africa	HM459556	HM459497	HM459497	HM459448
Empodium sp. nov.	Desmet 2979 (NBG)	N Cape, Aggeneys	South Africa	HM459557	HM459498	HM459498	HM459449
Hypoxidia maheensis F. Friedmann	leg. C. Küffer, s.n., 26. 11. 2002	Seychelles	Seychelles	HM459558	HM459499	HM459499	HM459450
∗*Hypoxidia rhizophylla* (Baker) F. Friedmann	leg. C. Küffer, s.n., 26. 11. 2002	Seychelles	Seychelles	HM459559	HM459500	HM459537	HM459451
Hypoxis angustifolia Lam.	cult. Kew Gardens 1973–2957	Cape Province	Tropical and Southern Africa, West Indian Ocean, Yemen	HM459560	HM459501	HM459501	HM459452
Hypoxis aurea Lour.	Kocyan AK426 (M)	Doi Suthep	Chiang Mai, Thailand	HM459562	HM459503	HM459503	HM459454
Hypoxis curtissii Rose	MW Chase 108 (NCU)	Duke Forest, North Carolina	USA	Z73702[a]	HM459504	HM459504	HM459455
Hypoxis decumbens L.	cult. Kew Gardens 1976–6038	Tres Cerros, Corrientes Province, Argentina	Mexico to tropical America	HM459563	HM459505	HM459505	HM459456
Hypoxis filiformis Baker	cult. BG Basel; Kocyan AK438 (Z/ZT)	–	South Africa	HM459561	HM459502	HM459502	HM459453
Hypoxis glabella R.Br.	MW Chase 2235 (K)	–	Australia, New Zealand	Y14989[a]	HM459506	HM459506	HM459457
Hypoxis hemerocallidea Fisch., C.A.Mey. & Avé-Lall.	cult. BG Berlin, 635807 (B)	–	Southern tropical and Southern Africa	HM459564	HM459507	HM459507	HM459458
∗*Hypoxis hirsuta* (L.) Coville	J.K. Wipf 2209 (M)	Texas	North America	HM459565	HM459508	HM459538	HM459459
Hypoxis hygrometrica Labill.	cult. BG Berlin, 43336 (B)	–	Australia	HM459566	HM459509	HM459509	HM459460
Hypoxis juncea Sm.	Whitten s.n. (K)	Florida	Southeast USA	HM459567	HM459510	HM459510	HM459461
Hypoxis occidentalis Benth.	s.n. (UWA); Kew DNA number 2234B	Wambyn Nature Reserve	Australia	HM459568	HM459511	HM459511	HM459462
Hypoxis parvula Baker	Singh 556 (NH)	Free State, Sentinel Peak	South Africa	HM639281	HM570029	–	HM639292
Hypoxis setosa Baker	29,156 (ZSS)	Cape Province	South Africa	HM459569	HM459512	HM459512	HM459463
Hypoxis villosa L.f.	cult. Kew Gardens 1972–3947	Cape Province	South Africa	HM459570	HM459513	HM459513	HM459464
Hypoxis sp.	cult. BG Bonn; T. Leyens & W. Lobin 126 (BONN)	–	Angola	HM459571	HM459514	HM459514	HM459465
∗*Molineria capitulata* (Lour.) Herb.	(1) Kocyan AK981228/1/01 (Z)	unknown, cult.	Tropical and subtropical Asia to north east Australia	(2) Z73701[a]	(1) HM459515	(1) HM459515	(1) HM459466
	(2) M.W. Chase 205 (NCU)						
Molineria crassifolia Baker	cult. Kunming Botanical Garden, s.n.	–	Nepal to China	HM459548	HM459516	HM459516	HM459467
Molineria latifolia (Dryand. ex W.T.Aiton) Herb. ex Kurz	Kocyan AK981023/1/03 (Z/ZT)	Sabah	Borneo	HM459550	HM459517	HM459517	HM459468
Molineria latifolia (Dryand. ex	Kocyan AK981019/1/01 (Z/ZT)	Sabah	Borneo	HM459549	HM459518	HM459518	–

W.T.Aiton) Herb. ex Kurz							
Molineria villosa Kurz	cult. BG Singapore s.n.; A. Kocyan AK971031/1/03 (Z/ZT)	–	Singapore	HM459551	HM459519	HM459519	–
Molineria sp. (aff. *M. latifolia*)	cult. BG Munich 98/3407; Bogner 2650 (M)	–	–	HM459552	HM459520	HM459520	HM459469
Pauridia longituba M.F.Thomps.	(1) Kocyan AK980624/1/01 (Z/ZT) (2) Snijman 1440 (NBG)	(2) W Cape, Vredenburg	South Africa	(1) HM459572	(1) HM459521[b] (2) HM459522	(1) HM459521[b] (2) HM459522	(1) – (2) HM459469
∗*Pauridia minuta* (L.f.) T.Durand & Schinz	Snijman 1812 (NBG)	W Cape, Rondeberg	South Africa	HM639282	HM570030	HM570030	HM639293
∗*Rhodohypoxis baurii* (Baker) Nel	cult. BG Basel, A. Kocyan AK990522/1/02 (Z/ZT)	–	South Africa	HM459573	HM459523	HM459523	HM459471
Rhodohypoxis baurii (Baker) Nel var. *baurii*	Singh 554 (NH)	Free State, Sentinel Peak	South Africa	HM639283	HM570031	HM570031	–
Saniella occidentalis (Nel) B.L.Burtt	Snijman 2059 (NBG)	N Cape, Hantamsberg	South Africa	HM639284	HM639301	HM639301	HM639294
∗*Saniella verna* Hilliard & B.L.Burtt	cult. BG Edinburgh 1997–3202	–	South Africa	HM459574	HM459524	HM459524	HM459472
Spiloxene alba (Thunb.) Fourc.	Snijman 2106 (NBG)	W Cape, Rondebosch	South Africa	HM459575	HM459525	HM459525	HM459473
Spiloxene aquatica (L.f.) Fourc.	Snijman 2113 (NBG)	W Cape, Rondebosch	South Africa	HM639285	HM639302	HM639302	HM639295
Spiloxene capensis (L.) Garside	UCI Arb. 728	–	South Africa	HM459576	HM459526	HM459526	HM459474
Spiloxene flaccida (Nel) Garside	Goldblatt & Manning 9585 (MO, NBG)	W Cape, Worcester	South Africa	HM459577	HM459527	HM459527	HM459475
Spiloxene gracilipes (Schltr.) Garside	Snijman 1753 (NBG)	W Cape, Langebaan	South Africa	HM639286	HM570032	HM570032	HM639296
Spiloxene linearis (Andrews) Garside	Snijman 1754 (NBG)	W Cape, Langebaan	South Africa	HM639287	HM639303	HM639303	HM639297
Spiloxene minuta (L.) Fourc.	E. Parker 452 (NBG)	W Cape, Elandsberg	South Africa	HM639288	HM570033	HM570033	HM639298
Spiloxene monophylla (Schltr. ex Baker) Garside	J.C. Paterson-Jones 913 (NBG)	W Cape, Hottentots Holland Mtns	South Africa	HM459578	HM459528	HM459528	HM459476
Spiloxene nana Snijman	Snijman 1865a (NBG)	N Cape, Oorlogskloof	South Africa	HM639289	HM570034	HM570034	HM639299
Spiloxene pusilla Snijman	Snijman 1860 (NBG)	W Cape, Matsikamma	South Africa	HM639290	–	–	–
Spiloxene schlechteri s.l. (Bolus) Garside	E. Parker 469 (NBG)	W Cape, Elandsberg	South Africa	HM639291	–	–	HM459477
Spiloxene scullyi (Baker) Garside	Harrower 3271 (NBG)	N Cape, Ploegberg	South Africa	HM459579	HM459529	HM459529	HM459478
Spiloxene trifurcillata (Nel) Fourc.	McMaster s.n. (NBG 208,084)	E Cape, Boschberg	South Africa	HM570028	HM639304	HM639304	HM639300
Outgroups							
Asteliaceae							
∗*Astelia alpina* R.Br.	A. Kocyan AK981010/1/01 (Z) MW Chase 1103 (K)	Tasmania	Tasmania/Australia	(1) HM459580	(1) HM459530	(1) HM459530	(2) HM459479
Astelia banksii A.Cunn.	MW Chase 1072 (K)	W Auckland, Karekare Beach	New Zealand	Y14983[a]	HM459531	HM459531	HM459480
Collospermum hastatum (Colenso) Skottsb.	(1) Adelaide BG 875,767 (2) –	(1) – (2) –	New Zealand	(1)Y14986[a]	(2) HM459532	–	–
Milligania stylosa (F.Muell. ex Hook.f.) F.Muell. ex Benth.	(1) A. Kocyan 981011/3/01 (Z) (2) MW Chase 511 (K)	(1) – (2) Tasmania, Adamson's Peak	Tasmania/Australia	(2) Z73693[a]	(1) HM459533	(1) HM459533	(2) HM459481
Blandfordiaceae							
∗*Blandfordia nobilis* Sm.	MW Chase 2835 (K)	–	New South Wales/Australia	Y14984[a]	HM459534	HM459534	HM459482
Blandfordia punicea Sweet	(1) AK981013/1/01 (Z/ZT) (2) M.W. Chase 519 (K)	Tasmania	Tasmania/Australia	(2) Z73694[a]	(1) HM459535	(1) HM459535	(2) HM459483
Lanariaceae							
∗*Lanaria lanata* (L.) T. Durand & Schinz	E.R. Orchard 342 (M)	–	South Africa	HM459581	HM459536	HM459536	HM459484

Herbarium acronyms follow the Index Herbariorum at http://sciweb.nybg.org/science2/IndexHerbariorum.asp. BG = botanical garden.

[a] Sequences taken from GenBank.

[b] Sequenced but not included in the final analysis.

region by comparing it with *Phalaenopsis aphrodite* ssp. *formosana* (GenBank accession AY916449) and descriptions of this region provided by Quandt et al. (2004).

2.4. Phylogenetic analyses

Phylogenetic analyses were performed using PAUP* 4.0b10 (Swofford, 2002) for parsimony reconstruction, GARLI v.1.0 (Zwickl, 2006) for maximum likelihood (ML) inference and MrBayes v3.1.2 for Bayesian inference (Ronquist and Huelsenbeck, 2003). All four regions were combined into a single matrix (in keeping with many recent studies; Moore et al., 2007). Prior to the final parsimony analysis, we performed a ratchet search using PRAP v2.0 (Müller, 2004: settings: 10 random addition cycles of 200 ratchet iterations using a simple-taxon-addition tree as starting point, tree-bisection-reconnection – TBR – branch swapping, steepest descent not in effect, and one tree held in memory; multiple tree – MulTree – option inactivated). This tree was used in the final heuristic analysis as the initial tree to search for potential shorter reconstructions (settings: TBR branch swapping, MulTrees and steepest descent options in effect with 100 replicates of random sequence addition, saving one tree for each replicate). Gaps were treated as missing data in all analyses. Internal support was assessed by non-parametric bootstrapping (Felsenstein, 1985). Parsimony bootstrap percentages (BP_P) were based on 10,000 replicates with the following heuristic settings (Müller, 2005): a simple-taxon-addition tree as the starting point, TBR swapping active, steepest descent not in effect, and one tree held in memory (MulTrees inactivated). Nodes with bootstraps of $\geqslant$85% are considered strongly supported here, whereas 75–84% is moderately and 50–74 is weakly supported (Chase et al., 2006).

The 24 models of molecular evolution implemented in MrModeltest 2.2 were tested for the ML and Bayesian analyses (Nylander, 2004). As GARLI v1.0 allows only a single model per analysis, we tested the combined dataset for the ML analysis. MrModeltest proposed the GTR + I+Γ model to be the most optimal under the Akaike information criterion (AIC). The number of generations for the ML analysis was set at five million, with the option for an early automated stop activated. This was met when no new and significantly better topology was detected. To obtain support for nodes, a ML heuristic bootstrap analysis (BP_{ML}) was performed with 100 replicates. For the Bayesian analysis, the data partitions were tested individually to determine the best substitution model for each. Under AIC, the GTR + I+Γ model was selected for *rbcL*, whereas the other regions were assigned the GTR+Γ model. For the combined Bayesian calculations, these models were applied to the different partitions accordingly. The Bayesian computations were run with four Markov chains, starting from random trees. The analyses were run for two million generations and sampled every 100th generation. Convergence was reached when the average standard deviation of split frequencies was below 0.01 and when log probabilities fluctuated in a minimum range. Accordingly, the first 4500–9200 trees obtained were deleted as part of the burn-in, and posterior probabilities (PP) were calculated from the remaining trees. The ML and Bayesian analyses were repeated three times from independent random starting trees.

3. Results

3.1. Phylogenetic analysis and tree topology

The *rbcL* matrix included 1363 base pairs (bp), which were easily aligned due to the absence of insertions-deletions (indels). In total, 207 positions were variable of which 133 were potentially parsimony informative (Table 3). The parsimony search produced more than 20,000 equally most parsimonious trees with a length of 261 steps, a consistency index (CI) of 0.61 and a retention index (RI) of 0.89. Alignment of the *trnL-F* region was more complicated due to indel regions (see below). The *trnL* intron matrix comprised 646 characters, of which 533 were retained in the analysis. Of those positions, 108 were variable and 51 were potentially parsimony informative. The parsimony search produced more than 20,000 equally most parsimonious trees with a length of 86 steps, a CI of 0.72 and a RI of 0.92 (Table 3). The *trnL* 3′ exon included 49 characters, of which only one was potentially parsimony informative (Table 3; no analysis performed). Of the 447 positions of the *trnL-F* spacer matrix 66 were variable and 34 potentially parsimony informative. The parsimony analysis produced 6786 most parsimonious trees with a length of 65 steps, CI of 0.68 and a RI of 0.89 (Table 3). Of the 1494 aligned positions in the *trnS-G* spacer, 991 were included in the analysis; 266 were variable of which 162 were potentially parsimony informative (Table 3). The analysis for this region produced 7166 equally most parsimonious trees with a length of 309 steps, a CI of 0.71 and a RI of 0.89 (Table 3). Analysis of the combined dataset produced 615 equally most parsimonious trees with a length of 739 steps, a CI of 0.66 and a RI of 0.88 (Table 3).

The parsimony, ML and Bayesian approaches for the combined dataset resulted in similar tree topologies with three well-supported major clades identified (Fig. 3). The *Curculigo* clade comprised species of *Curculigo*, *Molineria* and *Hypoxidia* (BP_P 100, BP_{ML} 100, PP 1.00). The second clade (*Hypoxis* clade) included *Hypoxis* p.p. – as in subgenus *Hypoxis* of Baker (as *Euhypoxis*; 1878) and section *Hypoxis* of Geerinck (1969) – and *Rhodohypoxis* (BP_P 100, BP_{ML} 100, PP 1.00). The *Pauridia-Empodium* clade comprised *Empodium*, *Pauridia*, *Spiloxene*, *Saniella* and two Australian species of *Hypoxis* belonging to Baker's (1878) subgenus *Ianthe* and Geerinck's (1969) section *Ianthe* (BP_P 85, BP_{ML} 81, PP 1.00). However, the relationships between the major clades received little or no support greater than BP 50.

The *Curculigo* clade is well resolved in all analyses, and most clades receive moderate to strong support; the species from the Seychelles, *Hypoxidia maheensis* and *H. rhizophylla* and *C. seychellensis* (BP_P 70, BP_{ML} 62, PP 0.96) are sister to the rest of the clade

Table 3
Description of the plastid data sets.

	Aligned length	Included characters	Constant characters	Variable but parsimony uninformative	Parsimony informative	CI	RI	Tree length
rbcL	1363	1363	1156	74	133	0.61	0.89	261
trnL intron	646	533	425	57	51	0.72	0.92	86
trnL (UAA) 3′ exon	49	49	43	5	1			
trnL-F spacer[a]	447	159	103	22	34	0.68	0.89	65
trnS-G	1494	991	725	104	162	0.71	0.89	309
combined		3095	2452	262	381	0.66	0.88	739

[a] The *trnL-F* spacer includes here the first three positions of the adjacent *trnF* gene, which were constant for all accessions.

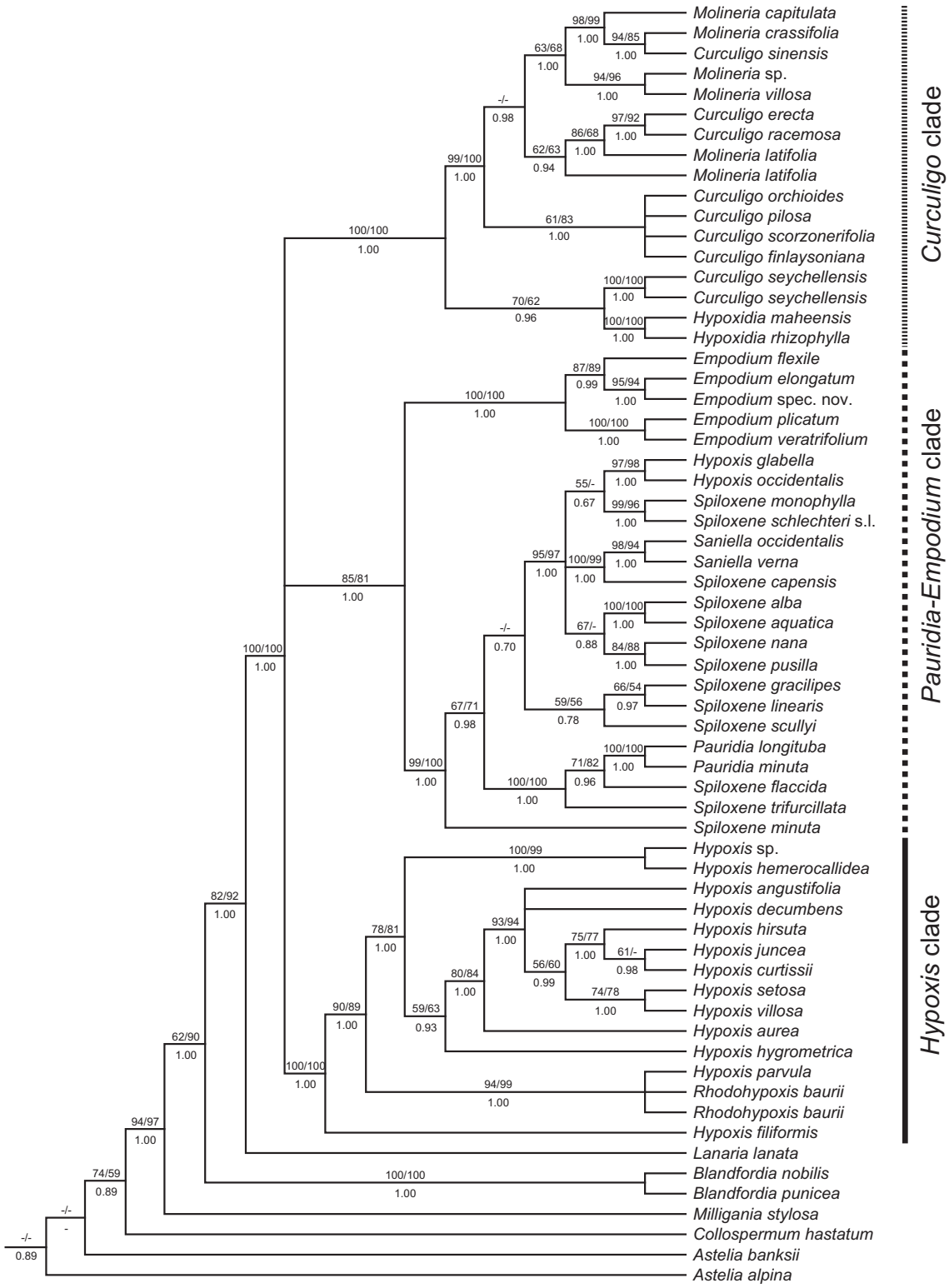

Fig. 3. Bayesian tree obtained from the analysis of the combined plastid dataset. Numbers above branches are BP_P/BP_{ML} and below branches PP.

(BP_P 99, BP_{ML} 100, PP 1.00). The remaining *Curculigo* species, including *C. finlaysoniana,* which is often placed in *Molineria*, are sister to *Molineria* s.s. (BP_P 61, BP_{ML} 83, PP 1.00). *Molineria* s.s. ($BP_P < 50$, $BP_{ML} < 50$, PP 0.98) is divided into three subclades, uniting the Chinese species, *M. crassifolia* and *Curculigo sinensis*, with *M. capitulata* (BP_P 98, BP_{ML} 99, PP 1.00) and also the two minor clades containing species from Borneo (*Curculigo erecta*, *Curculigo racemosa*, *M. latifolia*; BP_P 62, BP_{ML} 63, PP 0.94) and other parts of Asia (*M. villosa*, *Molineria* sp.; (BP_P 94, BP_{ML} 96, PP 1.00)). As currently circumscribed, *Curculigo* is polyphyletic.

Hypoxis could be defined as monophyletic only with inclusion of *Rhodohypoxis* and exclusion of two of the three Australian *Hypoxis* accessions (*H. glabella*, *H. occidentalis*). *Hypoxis filiformis* is sister to the rest of the main clade (BP_P 100, BP_{ML} 100, PP 1.00). The two accessions of *Rhodohypoxis baurii* fall together with *H. parvula* (BP_P 94, BP_{ML} 99, PP 1.00). The three North American *Hypoxis*

species sequenced, *H. hirsuta*, *H. curtissii* and *H. juncea*, form a moderately to well-supported clade (BP_P 75, BP_{ML} 77, PP 1.00) that is part of a clade (BP_P 78, BP_{ML} 81, PP 1.00) containing *Hypoxis* species from Africa (*H.* sp., *H. hemerocallidea*, *H. setosa* and *H. villosa*), Madagascar, and the Mascarene Islands (*H. angustifolia)*, Asia (*H. aurea*) and tropical America (*H. decumbens*).

Within the *Pauridia–Empodium* clade, *Empodium* (BP_P 100, BP_{ML} 100, PP 1.00) is sister to the *Pauridia* clade, which comprises a complex assemblage of the remaining genera (BP_P 99, BP_{ML} 100, PP 1.00). The analysis identified two well-supported clades within *Empodium*, (1) *E. plicatum* and *E. veratrifolium* (BP_P 100, BP_{ML} 100, PP 1.00) and (2) *E. flexile*, *E. elongatum* and *E.* sp. nov. (BP_P 87, BP_{ML} 89, PP 0.99). *Spiloxene* is polyphyletic. *Spiloxene minuta* is sister to the rest of the *Pauridia* clade (BP_P 99, BP_{ML} 100, PP 1.00), which consists of three subclades (BP_P 67, BP_{ML} 71, PP 0.98). The first subclade comprises the two species of *Pauridia* (*P. minuta* and *P. longituba*) together with *S. trifurcillata* and *S. flaccida* (BP_P 100, BP_{ML} 100, PP 1.00). The second subclade contains *S. gracilipes*, *S. linearis* and *S. scullyi* (BP_P 59 BP_{ML} 56, PP 0.78), which are sister to a third subclade (BP_P 95, BP_{ML} 97, PP 1.00). In turn, the third subclade contains three minor clades: (1) *S. aquatica* and *S. alba* (BP_P 100, BP_{ML} 100, PP 1.00), *S. nana* and *S. pusilla* (BP_P 84, BP_{ML} 88, PP 1.00), which are sisters (BP_P 67, $BP_{ML} < 50$, PP 0.88), (2) the two Australian *Hypoxis* species (*H. glabella*, *H. occidentalis*; BP_P 97, BP_{ML} 98, PP 1.00), which fall in most trees with two *Spiloxene* species (*S. monophylla*, and *S. schlechteri s.l.*; BP_P 99, BP_{ML} 96, PP 1.00), and (3) the smallest clade comprising *S. capensis* sister to the two species of *Saniella* (BP_P 100, BP_{ML} 99, PP 1.00).

3.2. Length differences in the trnL intron and the trnS-G spacer regions

Within Hypoxidaceae, the P8 region of the *trnL* intron ranged from 286 bp (*Spiloxene nana*) to 345 bp (*Hypoxis filiformis*). Outgroup taxa ranged from 279 bp (*Astelia alpina*) to 290 bp (*Lanaria lanata*). An unalignable hypervariable TA region of P8 was (with c. 74 positions) short and excluded from analysis. In the *trnL-F* spacer of the *Curculigo* and *Pauridia* clades, a large (c. 200 nucleotides) indel region was present. A similarly large indel (c. 177 bp) characterized the *Curculigo* and the *Hypoxis* clades in *trnS-G* (Fig. 4).

4. Discussion

4.1. Overall tree and effect of taxon sampling

The phylogenetic analyses identified three major clades: (1) *Curculigo* s.l. (including all species of *Curculigo* plus *Hypoxidia* and *Molineria*), (2) *Hypoxis* (*Hypoxis* s.s. plus *Rhodohypoxis*), and (3) *Pauridia–Empodium* (*Empodium*, *Hypoxis* p.p., *Pauridia*, *Saniella* and *Spiloxene*). This topology is in partial agreement with the groupings found by Rudall et al. (1998), namely (1) *Molineria*, *Empodium* and *Hypoxidia*, (2) *H. leptocarpa* (=*H. curtissii)* (North America) and *Rhodohypoxis*, and (3) *Pauridia*, *Spiloxene* and *Hypoxis glabella* from Australia. The only major discrepancy evident between the two analyses is in the position of *Empodium*, which was sister to the *Pauridia* clade in all our analyses. The differing topology of the 1998 tree was caused by editing errors in production of the sequence *E. veratrifolium*. There are several discrepancies between the old *rbcL* sequence of *Empodium* and our new sequence, and re-examination of the old electropherograms showed that incorrect basis were called in several places.

4.2. The Curculigo clade

Circumscription of *Curculigo* has been notoriously problematic in the taxonomic literature. Species that should be placed in *Molineria* are frequently placed in *Curculigo* and vice versa. The confusion is caused by the use of partly overlapping morphological characters that should ideally distinguish the genera from each other. One of the key characters used to define *Curculigo* is the beaked fruit arising from the persistent tissue that forms a rostrum between ovary and perianth. However, this character is also found in the *Molineria latifolia* complex, all species of *Empodium, Rhodohypoxis rubella* (Nel, 1914b), *R. incompta* and *R. thodiana* (Hilliard and Burtt, 1978), and *Spiloxene alba* (Thompson, 1978). Furthermore, it is also a diagnostic feature of *Saniella* (see below). In addition, this character occurs in *Curculigo seychellensis* of the Seychelles clade, which has an enormously elongated rostrum of up to 120 mm, whereas its sister genus *Hypoxidia* has none (Friedmann, 1984). Hence, this feature seems to have evolved several times independently within Hypoxidaceae and therefore cannot be used reliably to differentiate genera. The obviously homoplasious origin of the rostrum also raises the question of how best to classify *Curculigo seychellensis*. Our topology shows that retaining this species in *Curculigo* and maintaining *Hypoxidia* as a distinct genus would make *Curculigo* non-monophyletic, but inclusion within *Hypoxidia* would render generic circumscription even more difficult as each has clear floral and palynological (see below) apomorphies that contradict the value of such a change (Kocyan, unpubl. data). Hence, we propose the recognition of a new monotypic genus containing the taxon currently known as *Curculigo seychellensis* (Kocyan, in prep.), which reflects the independent evolutionary history of that taxon (MWC disagrees: he prefers to unify all members of the *Curculigo* clade in genus *Curculigo*). On the other hand, continued separation of *Curculigo* (excl. *C. seychellensis*) and *Molineria* based on insubstantial differences in their seeds and the shape of their rhizomes would serve only to obscure the clearly close relationship between these two groups (see below). Hence, to promote taxonomic utility we propose to unify *Curculigo* (excl. *C. seychellensis*) and *Molineria* (*Curculigo* is the older name), which should terminate a fruitless discussion that has persisted for over a hundred years.

4.3. The Pauridia–Empodium clade

The topology within the *Pauridia–Empodium* clade clearly indicates a close relationship between *Spiloxene*, *Saniella*, *Pauridia,* and the species of *Hypoxis* sect. *Ianthe* from Australia – the entire group here termed the *Pauridia* clade – even though they differ in floral morphology (Fig. 4). *Pauridia* (s.s) has only three functional stamens inserted on the perianth tube, with the stamens of the outer whorl reduced to staminodes; the filaments and style are united to form a short gynostemium, which is a relatively uncommon trait in flowers (Rudall and Bateman, 2002). Because of this character combination, *Pauridia* has been used in comparative studies addressing the evolution of the unique orchid flower, which shares characters such as reduced stamen number, absence of septal nectaries and presence of a gynostemium, the last a highly unusual feature among angiosperms. However, although Orchidaceae and Hypoxidaceae are both early-divergent Asparagales, they are not sisters. Orchid flowers are monosymmetric and possess a labellum. The reduction of anther number in *Pauridia* is clearly a parallel event unrelated to that in Orchidaceae, in which reduction is only on the adaxial side of the flower affecting both staminal whorls, whereas in *Pauridia* an entire stamen whorl is absent. Loss or reduction of entire whorls occurs in 38% of monocot families, and the relevant groups are pollinated by generalist insects or wind (Walker-Larsen and Harder, 2000). Of these, only five unrelated families have staminal whorls reduced to staminodes, and they are clearly not wind pollinated (Walker-Larsen and Harder, 2000, Kocyan, unpubl. data). The floral structure of *Pauridia* suggests that it is pollinated primarily by pollen-gathering insects

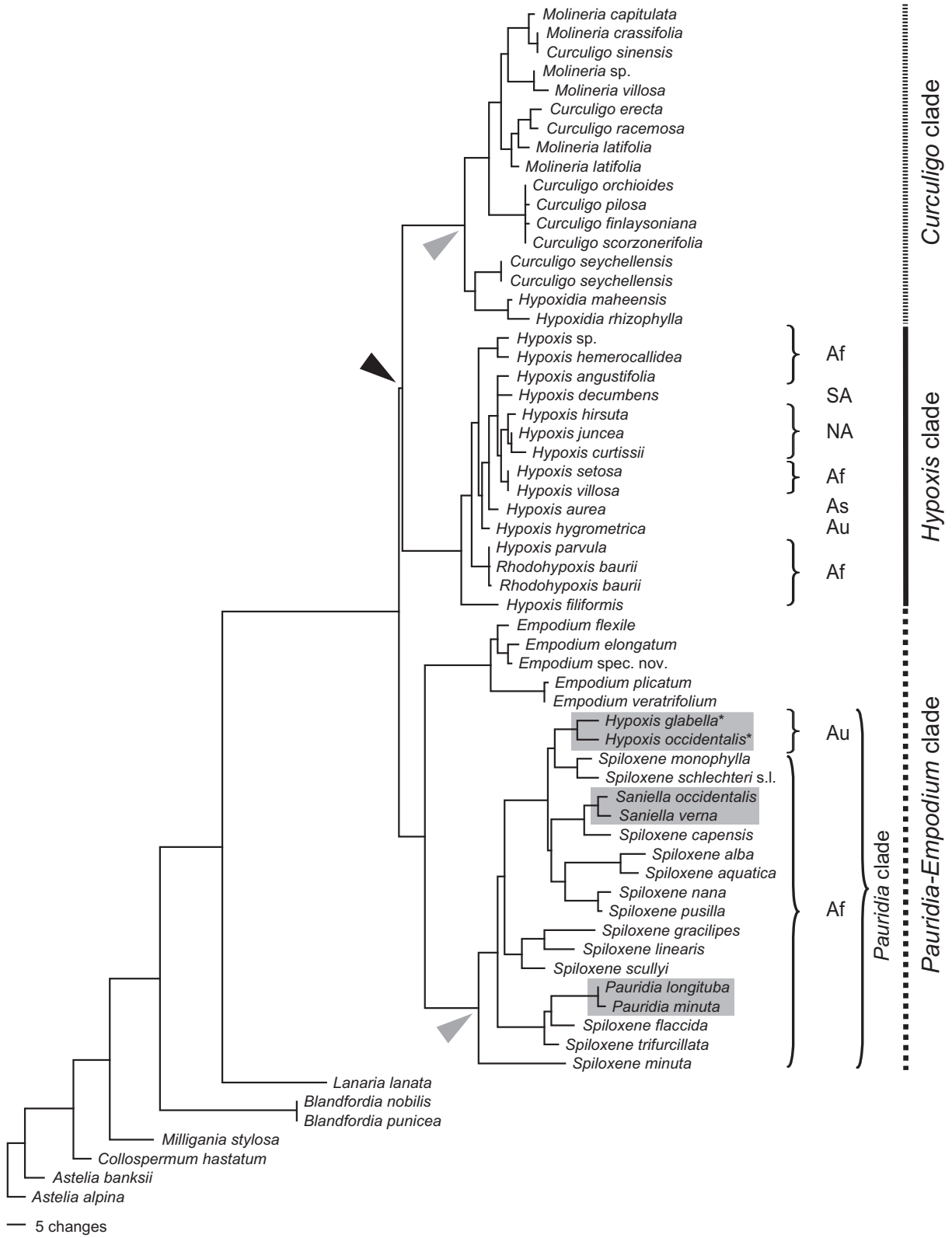

Fig. 4. One of 615 equally parsimonious trees of the combined dataset of Hypoxidaceae. The asterisks in the *Pauridia–Empodium* clade indicate the two species of *Hypoxis* of sect. *Ianthe* occurring in Australia. Grey rectangles indicate genera nested within *Spiloxene*. Arrow heads indicate indels: grey in the *trnL-F* spacer, black in the *trnS-G* spacer. Abbreviations: Af Africa, As Asia, Au Australia, NA North America, SA South America.

such as bees (Kocyan et al., unpubl. data). On the other hand, despite these floral specializations, several authors have proposed a close relationship between *Spiloxene* and *Pauridia* on the grounds that they share the similar vegetative and floral morphology (Hilliard and Burtt, 1978; Thompson, 1978; Burtt, 2000). Within Hypoxidaceae, the presence of a gynostemium is not exclusive to *Pauridia,* as the stamens are fused to the style in *Spiloxene pusilla* (Snijman, 2006). Hence, in the *Pauridia–Empodium* clade in general

there is conspicuous plasticity in the number of stamens and occurrence of a gynostemium.

The second genus found to be deeply nested within *Spiloxene* is *Saniella*. As currently circumscribed (Burtt, 2000), *Saniella* is distinguished from *Spiloxene* by an elongation between the subterranean ovary and the above ground style, stamens, and funnel-shaped perianth that results in a beaked fruit at maturity (Hilliard and Burtt, 1978). After expansion of the genus *Saniella* by Burtt (2000), the beaked ovary became the only feature to distinguish it from *Spiloxene*, thus rendering their separation questionable. Beaked ovaries also occur in some species of *Molineria,* in which some species have elongate ovaries and other taxa do not (see above), *Empodium,* in which species with short to long beaks occur (Thompson, 1978), and *Rhodohypoxis* (Hilliard and Burtt, 1978).

The third anomalous group found in the *Pauridia* clade comprises the two Australian *Hypoxis* species. In a recent flora treatment, these two species were placed in *Hypoxis* section *Ianthe* (Henderson, 1987), following Geerinck's (1969) interpretation that relegated the genus *Spiloxene* (= *Ianthe*, orthographic variant *Janthe*) of previous authors (Nel, 1914b; Fourcade, 1932; Garside, 1936) to sectional rank under *Hypoxis*. Our result confirms the proposals by Hilliard and Burtt (1978) and Manning et al. (2002) that *H. glabella* and its Australian allies in *Hypoxis* sect. *Ianthe* belong with the southern African species of *Spiloxene*. In summary, our phylogenetic findings for the *Pauridia* clade (*Pauridia*, *Saniella*, *Spiloxene*, *Hypoxis* sect. *Ianthe*) provide a strong argument for placing its members into an enlarged genus *Pauridia*, which has nomenclatural priority over *Spiloxene* and *Saniella*. The group shares mucilage canals associated with vascular bundles in the leaves (Rudall et al., 1998), and there are new palynological data available that provide further support for an expanded *Pauridia* (see below).

Although *Empodium* is sister to the large *Pauridia* clade, there is no reason to unify the two into a single genus. *Empodium* is the only genus of Hypoxidaceae that possesses an entirely unilocular ovary with three parietal placentas, and the pollen grains exhibit characters that clearly distinguish *Empodium* from its sister clade, *Pauridia* s.l. (see below; Kocyan et al., unpubl. res.). Furthermore, the inflorescence always consists of a single flower without bracts. There is no distinction into scape and pedicel (Thompson, 1978). In addition, De Vos (1949) described several features in the development of the ovary and ovule that are more specialized in *Empodium* – treated by her as *Forbesia* – than in *Spiloxene* and *Pauridia*. Lastly, *Empodium* is unique in having a persistent, broad and compact outgrowth on the seeds that Hilliard and Burtt (1978) described as strophiolate.

4.4. The Hypoxis clade

The *Hypoxis* clade is morphologically the most uniform group of Hypoxidaceae, even though it is widely distributed in Africa, the Americas, Australia, Asia, Madagascar, Mauritius, and Reunion. The only exception is the southern African *Rhodohypoxis*, which has sharply incurved claws at the base of the inner tepals, thus closing the perianth throat and concealing the short, small, biseriately inserted stamens and the compressed, triangular style. However, the species of *Rhodohypoxis* are sister to *H. parvula,* with which they can form natural hybrids (Hilliard and Burtt, 1978). Moreover, palynological research on the family has not revealed any substantial differences in pollen ornamentation between *Hypoxis* and *Rhodohypoxis* (Kocyan et al., unpubl. res.). Hence, we propose unifying *Rhodohypoxis* with *Hypoxis*. The enlarged genus *Hypoxis* shares in general the same indumentum of tufted or two-branched trichomes – only rarely solitary from a multicellular base – with *Curculigo* and *Hypoxidia* (Rudall et al., 1998). The hairs are found on the leaves and/or inflorescence and are often restricted to the backs of the outer tepals, at least along the midrib.

4.5. Biogeography

Our topology allows us to speculate on the biogeographical history of Hypoxidaceae and how the precursors of extant species could have spread between continents. At least in the case of *Hypoxis glabella* and *H. occidentalis,* long-distance dispersal between southern Africa and southern Australia is the only plausible explanation. The alternative hypothesis, separation by vicariance, would mean that *Hypoxis* sect. *Ianthe* entered Australia about 100 million years ago (mya) when the last land connection between southern Africa and Australia was about to open. However, the divergence time for Hypoxidaceae is estimated between 93 and 100 mya (Anderson and Janßen, 2009). This would indicate that the Australian *Pauridia* (=*Hypoxis* p.p.) lineage should represent a relatively early-branching node of Hypoxidaceae, a pattern that is not supported by our analysis. Similar disjunct distribution patterns have been reported in other angiosperms. *Crinum* (Amaryllidaceae) is hypothesized to have dispersed to Australia from Africa twice, once involving a trans-oceanic dispersal (Meerow et al., 2003). *Wurmbea* (Colchicaceae) is also hypothesized to have dispersed to Australia from Africa once and radiated thereafter, an event that has been explained by long-distance dispersal (Case et al., 2008). Another candidate for a similar long-distance dispersal scenario is *Bulbine* (Xanthorhoeaceae s.l.), with c. 45 species in Africa and five in Australia (Watson, 1987; Devey et al., 2006). *Caesia* (Asparagaceae) also occurs in both Africa and Australia.

The mechanisms by which such great distances have been achieved are widely debated. Whereas the transport capacities of seeds with dispersal structures such as the pappus hairs of Asteraceae are intuitively clear, wind transport of diaspores that lack such structures is more difficult to explain. One possibility for dust-like seeds such as those of orchids is that they are readily distributed by wind currents. Although the seeds of *Pauridia* (s.l.) are small and light (±0.5 mm by ±0.4 mm and ±0.4–1.0 mg), they are larger than those of orchids (Snijman, unpublished data). However, it remains possible that extreme wind events could transport these diaspores over huge distances. Recent work has begun to emphasize the role of the west wind drift (WWD), a circumpolar oceanic current associated with strong winds, in the dispersal of plants between southern Africa and southern Australia (Sanmartín and Ronquist, 2004; Waters, 2008; Bergh and Linder, 2009). The distribution of the non-African *Pauridia* species (=*Hypoxis* sect. *Ianthe*) occurring in mainland Australia, Tasmania, and New Zealand conforms well with the WWD hypothesis, as the WWD would be likely to direct material from the Cape of South Africa to southeastern Australia and Tasmania and possibly further east to New Zealand (Bayer et al., 2002; Waters and Roy, 2004). Should the WWD be the agent for such dispersal, the maximum age for this event would be about 35 mya, the estimated time at which the WWD became fully established after the separation of Antarctica from Australia (Stickley et al., 2004).

In contrast, a different situation is found in the larger *Hypoxis* group, which is distributed on all Southern Hemisphere continents (except Antarctica) and has outliers in North America and Asia. *Hypoxis* is most species-rich in southern Africa, but secondary centers of species richness occur to the north in sub-Saharan Africa (Wiland-Szymańska, 2001, 2009; Wiland-Szymańska and Nordal, 2006; Singh, 2006). *Hypoxis urceolata*, an otherwise East African species, is found at the western tip of the Arabian Peninsula and Socotra (Wood, 1997; Miller and Morris, 2004). The topology presented here hints at a southern African origin of the genus as suggested by the grade formed by the early-diverg-

ing lineages of *Hypoxis* (incl. *Rhodohypoxis*) composed of species that are endemic to this region. Also, it remains possible that *Hypoxis* has entered the Americas twice, because *H. decumbens* (South America) and *H. curtissii*, *H. hirsuta* and *H. juncea* (all North America) do not form a clade. Nevertheless, in this part of the *Hypoxis* tree the support is not high, and resolution is low. However, as we have sequenced only a fraction of the described species (c. 10%) a reliable *Hypoxis* phylogenetic analysis is impossible due to this low sampling and since reliable age estimates of the genus are currently unobtainable, it is difficult to draw more definite conclusions about vicariance or dispersal. Both scenarios are possible. *Hypoxis hygrometrica*, which is now found from Queensland to Tasmania, could have become established on the Australian landmass when the continent drifted away. *Hypoxis aurea* could potentially have drifted with the Indian subcontinent to spread subsequently into Asia. In a similar manner, *H. decumbens* could have inhabited South America. On the other hand, a variety of long-distance dispersal events could account for the observed geographic patterns. For example, the distribution of the crown group consisting of three African species and three North American species could be analogous with the patterns shown by some recently established sister-group relationships between tropical Africa and North America that have been attributed to long-distance dispersal, namely three *Jacquemontia* (Convolvulaceae) species from tropical Africa, the Caribbean, and Hawaii (Namoff et al., 2010) and West African *Crinum purpurascens* that is sister to the North American *Crinum* species (Kwembeya et al., 2007; Bjorå et al., 2009).

Despite differences in floral characters, the three species from the Seychelles form a clade and hence are a result of a small local radiation. *Curculigo* s.s., which probably consists of only four or five species of which four were sequenced here, occurs in tropical and subtropical areas of Africa, Asia, Australia and the Americas. They clearly form a clade but with surprisingly little intra-clade sequence variation. *Curculigo orchioides* also occurs in New Caledonia, which poses questions about the age of *Curculigo,* as its distribution on all Southern Hemisphere continents and tropical Asia could support a Gondwanan origin. However, the low levels of sequence variation found in this clade and the finding that New Caledonia was completely submerged during the Paleocene argue against such an ancient age (Pelletier, 2007).

4.6. Karyology and ploidy

Karyological information is almost complete at the generic level in Hypoxidaceae, although data for *Pauridia* s.s. and *Saniella* are lacking. However, only *Hypoxis* has been studied in depth karyologically, revealing that it is a complex genus in this respect with extremely variable chromosome numbers. Chromosomal counts of somatic cells range from 14, 16, 18, 22, 28, 32, 36, 53, 54, 55, 56, 67, 70, and 72 to 96 (up to 200), indicating that polyploidization, dysploidy and aneuploidy are putative speciation factors; higher numbered polyploids have been found to be apomictic (Nordal et al., 1985; Nordal, 1998; Zimudzi, 1994; Judd, 2000). A further noteworthy feature is that the species of *Hypoxis* occur on relatively short terminal branches (Fig. 4), which could be further evidence for the hybrid, apomictic or polyploid origin of several species (Zimudzi, 1996; Nordal, 1998). In addition, the basic chromosome number of *Hypoxis* is unclear; numbers published are 7, 8, 9, 11, or 14. *Rhodohypoxis*, which we regard here as part of *Hypoxis*, is unusual as it has $2n = 12$ (Saito, 1975). *Hypoxis filiformis*, which is sister to all other members of the *Hypoxis* clade, has $n = 7$ (Wilsenach and Papenfus, 1967). This means that the high chromosome number and variation in *Hypoxis* have their origin after the separation of the *Rhodohypoxis* clade. Although some members of the *Curculigo* clade are clearly distinct according to sequence and seed data, they share a basic chromosome number of 9 with diploids and tetraploids (Nordal, 1998). The isolated position of the Seychelles species is also supported by chromosomal data. *Curculigo seychellensis* has $2n = 20$ (Barigozzi, 1979), and *Hypoxidia rhizophylla* has $2n = 22$ (Baltisberger and Kocyan, 2010). The basic chromosome numbers of the *Pauridia* s.l. and *Empodium* clades are 6, 7, or 8, and the numbers in somatic cells are 12, 14, 16, and 28 (Snijman, 2000; Johnson, unpublished; Thompson, unpublished; Vosa, unpublished), which also hint at dysploidy and polyploidy.

Given the phylogenetic position of *Pauridia* s.s. and *Saniella* – both with unknown numbers – we expect a similar chromosomal number. In summary, a global trend for chromosome numbers between the clades of Hypoxidaceae is difficult to trace, even though most clades are characterized by defined chromosome numbers. Species within most (if not all) clades have undergone dysploidy, aneuploidy and polyploidization.

4.7. Habit

All Hypoxidaceae are herbs that arise from annual corms or tuberous to elongated rhizomes. The occurrence of cormous and rhizomatous features corresponds with the two large clades of Hypoxidaceae. Species of the *Pauridia–Empodium* clade have annual corms, whereas members of the *Hypoxis* clade and *Curculigo* clade are rhizomatous. Within the rhizomatous clade, three types can be distinguished: tuberous rhizomes of *Hypoxis* s.l., *Hypoxidia* and *Curculigo seychellensis*, thin rhizomes of *Molineria*, and vertically elongated tuberous rhizomes with fleshy roots of *Curculigo* s.s. The annual corms and tuberous rhizomes conform largely to species that occur in habitats marked by seasonal drought, when they persist as geophytes that produce small leaves during the favorable growth period. *Molineria*, *Hypoxidia* and *Curculigo seychellensis* occur in evergreen humid rainforests, so they are not dependent on storage organs to overcome adverse seasons and have – if any – small tuberous underground parts that give rise to evergreen, mostly large leaves.

4.8. Pollen structure

Palynology of Hypoxidaceae has so far received relatively little attention, so pollen information has been limited (Nordal, 1998). In general, pollen is (micro-) reticulate and columellate with one or two sulci (Erdtman, 1952; Thompson, 1979; Simpson, 1983; Furness and Rudall, 2003). However, in a recent study of all hypoxid genera, an increased level of palynological diversity has been discovered, although aperture number and surface structure appear to be consistent within the larger clades (Kocyan, Snijman, Halbritter, Hesse, unpubl. data). In short, the *Pauridia* clade has disulcate pollen with the exception of one of the Australian species, which is trisulcate, whereas *Empodium* is monosulcate. The species of *Hypoxis* s.l., *Curculigo* s.s. and *Molineria* included in the present study are exclusively monosulcate. The Seychelles species are either inaperturate or disulcate. Pollen surface ornamentation of all species is microreticulate, and most species of the *Pauridia* clade are microechinate.

4.9. Scents, pollinators and reward

Asparagales are typically characterized by the presence of septal nectaries; all the other families of the small astelioid clade of Asparagales (Asteliaceae, Blandfordiaceae, Lanariaceae) possess more or less prominent septal nectaries (Rudall, 1998; Kocyan and Endress, 2001b) with the exception of Hypoxidaceae. In contrast, flowers of Hypoxidaceae offer pollen as a reward (Nordal,

1998); no nectar has been detected, and the absence of nectaries is a diagnostic feature of the family (Judd, 2000; Judd et al., 2008).

Hypoxidia (*Curculigo* clade) contains the most intensely scented flowers of the family. The flowers emit an unpleasant, fetid odor, and together with their brownish red-colored tepals they exhibit the sapromyophilous syndrome (Fig. 1). Odor also occurs in some other genera: flowers of *Pauridia* (s.s) are either sweet (*P. minuta*) or acrid smelling (*P. longituba*), and they are visited by honeybees (*Apis mellifera*) and various flies in Calliphoidae, Syrphidae and Tachinidae (Markötter, 1936; Snijman, unpubl. data). These short-proboscid flies appear to be attracted to the minute quantities of exudate on the papillate hooks, which correspond with the position of the staminodes, in the clefts between the three stigmatic branches. Four species of *Spiloxene* emit a weak sweet scent, but all other *Spiloxene* species appear to be scentless. *Spiloxene* species are generally visited by pollen-collecting insects such as honeybees. However, an exception has been detected in the scentless *Spiloxene capensis*, which varies considerably in the size and coloring of its flowers. Forms with large flowers with darkly spotted centers belong to a guild of Cape plants in several families that have similarly marked flowers. They are typically pollinated by monkey beetles (Scarabaeidae: Hopliini), which depend primarily on these visual cues to locate flowers for pollen and as a place to mate (Fig. 1; Steiner, 1998; Johnson and Midgley, 2001). This is a remarkable example of convergent evolution in the South African flora, since *Ixia dubia*, the peacock species of *Moraea* (both Iridaceae), and *Gazania pectinata* (Asteraceae) reveal the same pattern. A lemon-like scent dominates in *Empodium*, and at least in some populations of *E. plicatum* the odor appears to be released by the anthers (Snijman, unpubl. data). In *Empodium,* honeybees have been seen collecting pollen and various beetles, including *Ceroctis capensis* (Meloidae), have been observed chewing the anthers and pollen (Markötter, 1936; Snijman, per. obs.). *Rhodohypoxis thodiana* (not sequenced) is the only species reported to have an odor in the *Hypoxis* clade (Hilliard and Burtt, 1978). The pollination biology of the complex flowers of *Rhodohypoxis* remains unknown. *Hypoxis*, *Curculigo*, and *Molineria* are in general scentless (A. Kocyan, pers.obs.). The yellow-flowered species of *Hypoxis* s.s. are generally visited by honeybees (Johnson and Anderson, 2002; Singh et al., 2007). Only recently, sweet scent was reported for *H. goetzei* and *H. fischerii* var. *zernyi*, the latter species being visited by ants (Wiland-Szymańska, 2009), but whether the ants are occasional pollinators as no obvious reward is offered or not must remain open here. Pollen-collecting *Trigona* bees have been observed on *Molineria* flowers (Kocyan and Endress, 2001b). Pollination information for *Curculigo* is unavailable, but because *Hypoxis* s.s. and *Molineria* have similar floral features, one may expect pollen-collecting bees as pollinators. No information on odor or pollination is available for *Saniella*.

Although our knowledge of scents and pollination systems of Hypoxidaceae is fragmentary, currently four pollination systems can be identified within the family: melittophily (bee), myophily (fly), sapromyophily (carrion fly), and cantharophily (beetle). Melittophily is ubiquitous. Cantharophilous systems are found solely in the southern African clades of Hypoxidaceae. Beetles may in part be the reason for the recurrent evolution of rostrum structures between the ovary and perianth; some beetles are known to feed on floral tissue and pollen and can easily destroy reproductive organs. By having a rostrum, beetles are less likely to damage the ovules, a scenario that could occur in *Empodium*. However, beetle pollination does not necessarily result in rostrate flowers, as it is also known in *Spiloxene capensis*. This species has exceptionally long anthers that possibly serve to offset the loss of some pollen to foraging insects. On the other hand, the beaked flowers and fruits of *Curculigo* and some *Molineria* species may present a different evolutionary scenario as their flowers are melittophilous. Nevertheless, Hilliard and Burtt (1973) emphasized the importance of the long ovary beak in providing protection to the ovary in both *Curculigo* and *Empodium*.

4.10. The P8 region of the trnL intron and the phylogenetic value of the trnS-G spacer

Quandt et al. (2004) investigated the molecular evolution of the *trnL* intron of land plants and in particular the development of the P8 stem-loop region. P8 contributes large parts of the total length of the *trnL* intron. However, substantial proportions of P8 are prone to exclusion from analysis due to non-alignability between larger clades of land plants, supporting the idea that elongation of P8 could be the result of several independent events between lineages. Published P8 lengths vary among angiosperms, from 91 bp (*Spinacia oleracea*, Amaranthaceae; Quandt et al., 2004.) to 639 bp (*Luisia curtisii*, Orchidaceae; Kocyan et al., 2008). However, extremely short P8 lengths – such as that of *Spinacia oleracea* – could be the result of secondary losses of large parts (Quandt et al., 2004). The lengths measured in this paper are 286–345 in the ingroup taxa and 279–290 in the outgroup taxa, representing about the average size known for this region, at ca. 250 bp for angiosperms (estimated from Quandt et al., 2004). In contrast with phylogenetic analyses at higher systematic ranks, in which almost the whole P8 region is usually excluded due to non-alignability, we were only constrained to exclude a short satellite-similar stretch of the P8 section that was characterized by hypervariable TA repeats. The systematic value of the excluded region is disputed, and the only known case to date that proposed some value was in the orchid genus *Aerides*, in which the three sections are characterized by P8 length classes (Kocyan et al., 2008). In Hypoxidaceae, we could not detect a correlation between P8 lengths and the three main clades or the grouping within clades.

In contrast to our expectations, the *trnS-G* region is easy to amplify, and no sign of inversion of the *trnS-G* region at the priming sites was detected. This situation differs from rice, where an inversion does not allow amplification (Hiratsuka et al., 1989). Our *trnS-G* matrix contained almost double the number of parsimony informative sites as the *trnL* intron and *trnL-F* spacer (162 vs. 86; Table 3), although these were difficult to align. Approximately the same ratios were found in e.g. *Satyrium* (Orchidaceae; van der Niet and Linder, 2008) and *Lathyrus* (Fabaceae; Kenicer et al., 2005). The information content of *trnS-G* is even higher than that of the *rbcL* gene, with 133 informative positions. Thus, the *trnS-G* region of Hypoxidaceae represents a valuable contribution to the easily amplifiable plastid regions, and it may be found to contribute important phylogenetic information in other groups of monocots.

4.11. Conclusions and prospects

The analyses presented here allow us to distinguish three main clades of Hypoxidaceae. The *Pauridia–Empodium* clade has a southern African center of diversity with an exceptional occurrence in Australia that is best explained by a long-distance dispersal event, whereas the distributions of the *Hypoxis* and *Curculigo* clades are more difficult to explain. Although the three main clades receive strong support, their interrelationships remain unclear. However, morphological arguments (rhizomes versus annual corms) may favor a closer relationship between the *Hypoxis* and *Curculigo* clades. Hence, we intend to further resolve relationships among clades of Hypoxidaceae by sequencing DNA regions of the two other genomes (mitochondrial and nuclear). Having a more complete phylogenetic reconstruction at hand, an age estimate for the family and

their clades, despite the absence of fossils, should be possible when calibrated by the ages of oceanic islands with known ages and endemic Hypoxidaceae and Asteliaceae or with more global analyses including as well taxa with fossils.

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