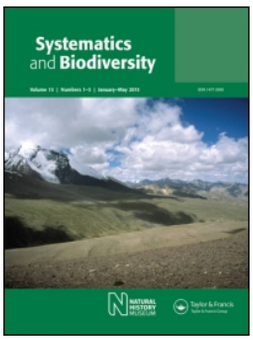

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Research Article

Phylogenetic relationships in *Bulbostylis* (Abildgaardieae: Cyperaceae) inferred from nuclear and plastid DNA sequence data

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Previous molecular phylogenetic analyses of the family Cyperaceae based on *rbc*L sequences showed *Bulbostylis* as paraphyletic, with *B. atrosanguinea* and *B. hispidula* forming a clade with *Nemum spadiceum*. On the contrary, phylogenetic analyses of the tribe Abildgaardieae based on nuclear (ITS ribosomal region) and plastid sequences (*trn*L-F region) showed *Bulbostylis* as monophyletic, although they only incorporated four species of *Bulbostylis* and none of *Nemum*. In this work, we presented a phylogenetic hypothesis of *Bulbostylis* based on a comprehensive sampling, including species from different continents for the first time. New sequences of *Abildgaardia*, *Crosslandia*, *Fimbristylis*, and *Nemum* were included to test the monophyly of *Bulbostylis*. In total, 84 sequences of both ITS and *trn*L regions were generated. Analyses were performed using Bayesian inference, maximum likelihood, and parsimony. Ancestral state reconstruction was performed using ML, MCMC, and parsimony methods. In all analyses, *Bulbostylis* resulted paraphyletic as *Nemum atracuminatum* is nested within it. Most American species of *Bulbostylis* grouped together, but relationships amongst them appeared poorly resolved. Ancestral state reconstructions of native distribution suggest an African ancestor of *Bulbostylis*, with at least three introduction independent events of the species in America. Morphological diagnostic characters such as the 'style base permanence or detachment from the ripe achene', and the 'micromorphological patterns of the achene surface' are homoplastic in this phylogenetic context, and therefore unsuitable to propose infrageneric groupings within the *Bulbostylis*.

Key words: Bayesian inference, ITS, maximum likelihood, molecular phylogenetic analysis, parsimony, *trn*L intron

Introduction

The genus *Bulbostylis* Kunth nom. cons. is included in tribe Abildgaardieae (sensu Goetghebeur, 1998), together with *Abildgaardia* Vahl, *Crosslandia* W. Fitzg., *Fimbristylis* Vahl, *Nelmesia* Van der Veken, and *Nemum* Desv. ex Ham. *Bulbostylis* has *c.* 200 species (Govaerts et al., 2016) with pantropical distribution, mainly concentrated in South America and tropical Africa (Goetghebeur, 1998). In previous studies, *Bulbostylis* has been characterized by long hairs at the mouth of the leaf sheath, spikelets with deciduous glumes, 3(-2)-fid styles with their base thickened and persistent (rarely deciduous) on the ripe fruit, trigonous (rarely biconvex) achene with the pericarp cells vertically elongated and commonly with silica bodies (= silicophytoliths), and *Bulbostylis*-type embryo (Barros, 1947; Goetghebeur, 1998; Goetghebeur & Coudijzer, 1984, 1985; Gonzalez & López, 2010; Guaglianone, 1970; Kral, 1971; Kral & Strong, 1999; Lye, 1971; Pedersen, 1969; Svenson, 1957; Van der Veken, 1965).

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Based on morphological characters some authors have attempted to establish infrageneric categories for this complex genus of Cyperaceae. Clarke (1908) for a reduced number of species proposed infrageneric groupings for *Bulbostylis* according to inflorescence configuration and the number of stigmatic branches. These groupings are not currently accepted, since most species of *Bulbostylis* have a trifid style and inflorescences are highly variable (e.g., *B. juncoides* (Vahl) Kük. ex Herter and *B. communis* M.G. López & D.A. Simpson), even amongst individuals of the same population. López (2012) and López and Gonzalez (2017) in their taxonomic study of *Bulbostylis* for the southern part of South America observed that the most stable characters for distinguishing *Bulbostylis* species are related to micromorphology of fruit surface; particularly, the presence or absence of silicophytoliths in the exocarp cells. Based on these characters, López (2012) and López and Gonzalez (2017) suggest informal groupings for *Bulbostylis* (Table 1).

Up to now, no molecular phylogenetic studies have attempted to clarify relationships in *Bulbostylis*. Phylogenetic analyses of the Cyperaceae family based on *rbc*L sequence data (Muasya et al., 2009; Muasya, Simpson, Chase, & Culham, 1998; Simpson et al., 2007) have included a limited sampling of this genus. Based only on two species of *Bulbostylis* [*B. hispidula* (Vahl) R.W. Haines and *B. atrosanguinea* (Boeckeler) C.B. Clarke], these studies showed *Bulbostylis* as paraphyletic, with *B. atrosanguinea* and *B. hispidula* forming a clade with *Nemum spadiceum* (Lam.) Desv. ex Ham. On the other hand, phylogenetic analyses of the tribe Abildgaardieae based on nuclear (ITS ribosomal region) and plastid sequences (*trn*L-F region) (Ghamkhar, Marchant, Wilson, & Bruhl, 2007) showed *Bulbostylis* as monophyletic; however this phylogenetic hypothesis included only four species of *Bulbostylis* and none of *Nemum*.

In this work, to clarify the evolutionary history of the *Bulbostylis* and to test the validity of the infrageneric groups proposed by López (2012) and López and Gonzalez (2017), we increased the sampling of *Bulbostylis* including other Abildgaardieae species and used molecular data based on the nuclear internal transcribed spacer (ITS) region and the plastid *trn*L region. Additionally, we discussed morphological features and biogeographic distribution in the current phylogenetic context.

Materials and methods

Taxon sampling

Our sampling consists of 39 species of *Bulbostylis*, and 18 species representing six other genera of tribe Abildgaardieae (*sensu* Muasya et al., 2009; where the genera *Actinoschoenus* Benth, *Arthrostylis* R. Br., and *Trachystylis* S. T. Blake of the tribe Arthrostylideae were transferred into Abildgaardieae). Sequences to represent these last genera were selected from Ghamkhar et al. (2007), Muasya et al. (1998), 2009), and Simpson et al. (2007). *Actinoschoenus composita* is here included following results by Ghamkhar et al. (2007) where this species is clearly apart from *Fimbristylis*; however, the valid name would be *Fimbristylis composita* until a new combination is accepted. Additionally, we included seven putative new species of *Bulbostylis*, whose names are being published independently of this phylogeny (Ardissone ined.). A complete list of taxa, voucher information, their locations, and GenBank accession numbers for DNA sequences are shown in Table 1. A total of 84 out of 117 sequences were generated in this work; 33 were downloaded from GenBank. Plant material was collected in the field and dried in silica gel (voucher specimens stored at SF, CTES, ICN or FLOR) or amplified from herbarium specimens (Table 1).

DNA extraction, amplification, and sequencing

Total DNA was extracted from silica-dried culms and leaves using a modified CTAB protocol by Doyle and Doyle (1987), or from herbarium material using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The internal transcribed spacer region comprising the ITS1-5.8S-ITS2 nuclear ribosomal DNA (ITS) was amplified by the polymerase chain reaction (PCR), using primers 17SE and 26SE designed by Sun, Skinner, Liang, and Hulbert (1994). The cpDNA *trn*L UAA intron was amplified using primers C and D by Taberlet, Gielly, Pautou, and Bouvet (1991). Reactions were performed on a TGradient Thermocycler (Biometra, Göttingen, Germany) in 25 μL volume containing template DNA, 0.25 μM of each primer, 25 μM dNTP, 5 mM MgCl2, 1× buffer and 1.5 units of Taq polymerase. PCR amplifications for ITS were carried out under the following conditions: initial denaturation (94°C for 3 min) followed by 38 cycles of denaturation (94°C for 30 s), annealing (53°C for 1 min) and elongation (72°C for 90 s) and a final elongation at 72°C for 10 min. Thermal cycling parameters for *trn*L were: an initial denaturation step (3 min, 94°C) followed by 38 cycles of denaturation (30 s, 94°C), annealing (1 min, 50°C) and elongation (90 s, 72°C) and a termination step of 10 min at 72°C. In species for which these parameters were unsuccessful, variation in the annealing temperature was used (48–55°C). Additionally, PCR additives and enhancing agents [bovine serum albumin, dimethyl sulfoxide, Q of the Taq PCR Core Kit (Qiagen)] were used to increase the yield, specificity, and consistency of PCR reactions. To confirm the presence of a simple product of the

Table 1. List of the samples used in the molecular analysis with species names, voucher information (collector, number, and where the specimen is housed), country of collection, and GenBank accession for DNA sequences of ITS and *trn*L regions (**bold** indicates new accession); a dash (–) indicates missing data; *treated as in Ghamkhar et al. (2007); **Bulbostylis species included in López (2012), and their informal infrageneric grouping; n.a. = not applicable data; *sp1–sp7* are unpublished species included in this study; *sp8* is an unpublished species included in Ghamkhar et al. (2007).

Taxon	Voucher (Herbarium Code)	Provenance	ITS	*trn*L intron	Group**
OUTGROUP					
Abildgaardia ovata (Burm. f.) Kral	V. Klaphake 1410 (NSW)	Australia	AY506758	AY506708	n.a.
Abildgaardia ovata (Burm. f.) Kral	A. Reutemann 173 (SF)	Argentina	**MG844187**	**MG844229**	n.a.
Abildgaardia schoenoides R.Br.	K. L. Clarke 70 (NE)	Australia	AY506761	AY506706	n.a.
*'Actinoschoenus composita'** (*Fimbristylis composita* Latz)	K. L. Clarke 213 (NE)	Australia	AY506755	AY506702	n.a.
Arthrostylis aphylla R.Br.	K. L. Clarke 183 (NE)	Australia	AY506757	AY506700	n.a.
Crosslandia setifolia W.Fitzg.	K. L. Clarke 246 (NE)	Australia	AY506768	AY506718	n.a.
Crosslandia setifolia W.Fitzg.	Cowie 13158 (DNA)	Australia	**MG844188**	**MG844230**	n.a.
Fimbristylis arnhemensis Latz	K. L. Clarke 177 (NE)	Australia	AY506776	AY506722	n.a.
Fimbristylis autumnalis (L.) Roem. & Schult.	L. Lucero 28 (SF)	Argentina	**MG844189**	**MG844231**	n.a.
Fimbristylis bisumbellata (Forssk.) Bubani	K. L. Clarke 107 (NE)	Australia	AY506778	AY506724	n.a.
Fimbristylis cinnamometorum (Vahl) Kunth	J. J. Bruhl 2058 (NE)	Australia	AY506772	AY506721	n.a.
Fimbristylis complanata (Retz.) Link	A. Reutemann 37 (SF)	Argentina	**MG844190**	**MG844232**	n.a.
Fimbristylis cymosa R.Br.	K. L. Wilson 10041 (NSW)	Australia	AY506798	AY506750	n.a.
Fimbristylis dichotoma (L.) Vahl	M. G. López 376 (CTES)	Argentina	**MG844191**	–	n.a.
Fimbristylis dichotoma (L.) Vahl	R. Ardissone 415 (ICN)	Brazil	–	**MG844233**	n.a.
Fimbristylis dichotoma (L.) Vahl	A. Reutemann 103 (SF)	Argentina	–	**MG844234**	n.a.
Fimbristylis laxiglumis Latz	K. L. Clarke 106 (NE)	Australia	AY506785	AY506736	n.a.
Fimbristylis pterigosperma R.Br.	K. L. Clarke 118 (NE)	Australia	AY506794	AY506729	n.a.
Fimbristylis spadicea (L.) Vahl	A. Reutemann 76 (SF)	Argentina	**MG844192**	**MG844235**	n.a.
Fimbristylis squarrosa Vahl	A. Reutemann 63 (SF)	Argentina	**MG844193**	**MG844236**	n.a.
Fimbristylis tetragona R.Br.	K. L. Clarke 173 (NE)	Australia	AY506799	AY506746	n.a.
Nemum atracuminatum Larridon, Reynders & Goetgh.	S. Lisowski 5003 (PRE)	Zaire	**MG844228**	**MG844270**	n.a.
INGROUP					
Bulbostylis aspera M.G.López	R. Ardissone 117 (FLOR)	Brazil	**MG844194**	**MG844237**	1
Bulbostylis barbata (Rottb.) C.B.Clarke	K. L. Clarke 113 (NE)	Australia	AY506764	AY506709	n.a.
Bulbostylis capillaris (L.) C.B.Clarke	R. Ardissone 384 (ICN)	Brazil	**MG844195**	**MG844238**	n.a.
Bulbostylis communis M.G.López & D.A.Simpson	A. Reutemann 23 (SF)	Argentina	–	**MG844239**	1
Bulbostylis communis M.G.López & D.A.Simpson	A. Reutemann 181 (SF)	Argentina	**MG844196**	–	1
Bulbostylis conifera (Kunth) C.B.Clarke	Dias-Melo 116 (NY)	Brazil	–	**MG844240**	n.a.
Bulbostylis consanguinea (Kunth) C.B.Clarke	R. Ardissone 197 (FLOR)	Brazil	**MG844197**	**MG844241**	3
Bulbostylis contracta (Kük.ex Osten) M.G.López & D.A.Simpson	R. Ardissone 269 (FLOR)	Brazil	**MG844198**	–	1
Bulbostylis cruciformis (Lye) R.W.Haines	Faden 74/776 (PRE)	Kenya	**MG844199**	**MG844242**	n.a.
Bulbostylis densa (Wall.) Hand.-Mazz.	R.P. Glen 720 (PRE)	South Africa	**MG844200**	–	n.a.
Bulbostylis densa (Wall.) Hand.-Mazz.	V. Klaphake 1411 (NSW)	Australia	AY506763	AY506710	n.a.
Bulbostylis funckii (Steud.) C.B.Clarke	E. H. Roalson 1384 (RSA)	Mexico	AF190616	–	1
Bulbostylis hirtella (Schrad. Ex Schult.) Nees ex Urb.	R. Ardissone 200 (FLOR)	Brazil	**MG844201**	**MG844243**	3
Bulbostylis hispidula (Vahl) R.W.Haines	L. Smook 9956 B (PRE)	Namibia	**MG844202**	**MG844244**	n.a.
Bulbostylis junciformis (H.B.K.) C.B.Clarke ex S.Moore	R. Ardissone 189 (FLOR)	Brazil	**MG844203**	**MG844245**	2
Bulbostylis juncoides (Vahl) Kük. Ex Herter	A. Reutemann 153 (SF)	Argentina	**MG844204**	**MG844246**	3
Bulbostylis loefgrenii (Boeck.) Prata & López	R. Ardissone 186 (FLOR)	Brazil	**MG844205**	–	3
Bulbostylis loefgrenii (Boeck.) Prata & López	A. Reutemann 158 (SF)	Argentina	–	**MG844247**	3
Bulbostylis macra (Ridl.) C.B.Clarke	C.J. Kayombo 1725 (PRE)	Tanzania	**MG844206**	**MG844248**	n.a.
Bulbostylis mucronata C.B.Clarke	D.S. Hardy 6511 A (PRE)	Namibia	**MG844207**	**MG844249**	n.a.
Bulbostylis paradoxa (Spreng.) Lindm.	R. Ardissone 372 (ICN)	Brazil	**MG844208**	**MG844250**	1

(*continued*)

Table 1. (*Continued*)

Taxon	Voucher (Herbarium Code)	Provenance	ITS	*trn*L intron	Group**
Bulbostylis pseudoperennis Goetgh.	Malaisse 317 (PRE)	Zaire	**MG844209**	**MG844251**	n.a.
Bulbostylis pusilla (Hochst. ex A.Rich.) C.B.Clarke	Retief 1545 (PRE)	Namibia	**MG844210**	**MG844252**	n.a.
Bulbostylis rugosa M.G.López	A. Reutemann 85 (SF)	Argentina	**MG844211**	**MG844253**	1
Bulbostylis scabra (J.Presl & C.Presl) C.B.Clarke	A. Reutemann 100 (SF)	Argentina	**MG844212**	**MG844254**	1
Bulbostylis scabricaulis Cherm.	C. Reid 1728 (PRE)	South Africa	**MG844213**	**MG844255**	n.a.
Bulbostylis scleropus C.B.Clarke	A. de Castro 478 (PRE)	South Africa	**MG844214**	**MG844256**	n.a.
Bulbostylis sphaerocephala (Boeck.) Lindm.	A. Reutemann 161 (SF)	Argentina	**MG844215**	**MG844257**	2
Bulbostylis sphaerolepis (Boeck.) Beetle	A. Reutemann 169 (SF)	Argentina	**MG844216**	**MG844258**	3
Bulbostylis stenocarpa Kük.	R. Ardissone 162 (FLOR)	Brazil	**MG844217**	**MG844259**	3
Bulbostylis striatella C.B.Clarke	J. J. Bruhl 2084 (NE)	Australia	AY506765	AY506711	
Bulbostylis subtilis M.G.López	A. Reutemann 156 (SF)	Argentina	**MG844218**	**MG844260**	3
Bulbostylis trabeculata C.B.Clarke	C. J. Ward 11981 (PRE)	Namibia	**MG844219**	**MG844261**	n.a.
Bulbostylis wanderleyana Prata & M.G.López	A. Reutemann 159 (SF)	Argentina	**MG844227**	**MG844269**	3
Bulbostylis sp1	R. Ardissone 370 (ICN)	Brazil	**MG844220**	**MG844262**	n.a.
Bulbostylis sp2	R. Ardissone 196 (FLOR)	Brazil	**MG844221**	**MG844263**	n.a.
Bulbostylis sp3	R. Ardissone 135 (FLOR)	Brazil	**MG844222**	**MG844264**	n.a.
Bulbostylis sp4	R. Ardissone 382 (ICN)	Brazil	**MG844223**	**MG844265**	n.a.
Bulbostylis sp5	R. Ardissone 27 (FLOR)	Brazil	**MG844224**	**MG844266**	n.a.
Bulbostylis sp6	R. Ardissone 352 (FLOR)	Brazil	**MG844225**	**MG844267**	n.a.
Bulbostylis sp7	R. Ardissone 418 (ICN)	Brazil	**MG844226**	**MG844268**	n.a.
Bulbostylis sp8	K. L. Clarke 184 (NE)	Australia	AY506766	AY506713	n.a.

amplified DNA, PCR products were electrophoresed using a 1% agarose gel in a 1× Tris-acetate-EDTA (TAE) buffer, stained with GelRed™ Nucleic Acid Gel Stain (BIOTIUM) and visualized under UV light. PCR products were cleaned and sequenced by Macrogen, Inc. (Seoul, Korea) using the ABI PRISM BigDye Terminator Cycle Sequencing Kits with AmpliTaq DNA polymerase (Applied Biosystems, Seoul, Korea). Both forward and reverse strands were sequenced; single-pass sequencing was performed on each template using the same primers used for PCR reactions. Editing and assembling of sequences was conducted using the ChromasPro v.1.7.5 program (Technelysium Pty, Ltd), which was also used for checking the quality of sequences. Sequence alignments were performed using Muscle v.3.8 (Edgar, 2004), and inspected by eye using BioEdit v.7.2.0 (Hall, 1999).

Phylogenetic analyses. Phylogenetic analyses were conducted for individual (ITS or *trn*L intron) and combined ITS+*trn*L intron datasets under parsimony (P), Maximum likelihood (ML), and Bayesian inference (BI). The P analyses were performed using TNT v.1.1 program (Goloboff, Farris, & Nixon, 2003, 2008) under heuristic searches with 1,000 random addition sequences and Tree Bisection and Reconnection branch swapping (TBR), retaining a maximum of 2 trees in each replicate. The optimal trees obtained were then submitted to a new round of TBR branch swapping to completion. All characters were equally weighted, treated as unordered, and gaps were scored as missing data. Prior to heuristic searches, all uninformative characters were deactivated. To evaluate the relative support for individual clades, bootstrap analysis (Felsenstein, 1985) was performed using a total of 10,000 replicates. The ML analyses were carried out using RAxML v.8.2.4 (Stamatakis, 2014), conducted on the CIPRES Science Gateway Web server (https://www.phylo.org). We selected the GTRGAMMA model and the rapid bootstrapping algorithm and ran 1,000 bootstrap replicates. Bootstrap values (BS) over 50% are reported for both P and ML analyses. For BI, we used the Bayesian Markov Chain Monte Carlo (MCMC) method, implemented in MrBayes v.3.2 (Ronquist et al., 2012) on CIPRES Science Gateway. Models of nucleotide substitution were determined by the Akaike Information Criterion (AIC) using jModelTest v.2.1.6 (Darriba, Taboada, Doallo, & Posada, 2012); the model selected was different for each partition analysed; ITS1: TIM2ef+G, 5.8S: K80, ITS2: SYM+G, and *trn*L: TIM3+G. Two simultaneous runs of 10 million generations, with random starting trees, were performed and sampled every 1,000 generations. The first 2,500 trees of each run (25%) were discarded as burn-in. The remaining trees (15,002) were combined to obtain the 50% majority rule consensus tree. Posterior probability (PP) values > 0.65 were recorded on the tree.

Ancestral state reconstruction. The native distribution was coded as a three-state character (0, America; 1, Africa; 2, Asia+Oceania). The character state assignment was based on Govaerts et al. (2007), herbarium specimens, and the information available in the portals Tropicos (http://www.tropicos.org) and e-Monocot (http://families.e-monocot.org).

Ancestral state reconstruction was performed using ML, MCMC, and parsimony methods. Parsimony reconstruction was carried out in Mesquite 3.04 software (Maddison & Maddison, 2015) using the 'trace character history' option (character states were treated as unordered). The reconstruction with ML and MCMC methods was performed using the 'multistate' module in BayesTraits software (Pagel, Meade, & Barker, 2004). In order to choose a model for MCMC runs, an initial ML run was carried out with 15,002 trees to set the range of hyperprior (0–779) and seed an exponential distribution. Many exploratory chains had to be run to establish a correct rate deviation parameter (ratedev = 60) for the characters in order to achieve an approximate 20–40% acceptance rate. Once all the MCMC parameters were set, ancestral states were estimated using the Most Recent Common Ancestor (MRCA) command, which finds the proportion of the likelihood associated with each of the possible states at the nodes. The analysis was run for 10 million generations, discarding the first million as burn-in and sampling every 1,000th generation to ensure independence. Convergence and ESS were checked with Tracer v1.5.0 (Rambaut & Drummond, 2007). The 'fossil' command was performed to test whether a particular state was significantly more likely at each node. The results of these MCMC runs were tested by estimating Bayes factor (BF), an approach based on smoothed estimates of marginal likelihood analysed with Tracer v1.5.0 (Rambaut & Drummond, 2007), which applies the method used by Newton and Raftery (1994) with modifications by Suchard, Weiss, and Sinsheimer (2001).

Results

The data matrix of the entire ITS region consisted of 59 sequences and 589 aligned characters, of which 219 were parsimony-informative; the length of the ITS sequences within the ingroup varied from 507 base pairs (bp) (*B. sphaerocephala*) to 545 bp (*B. paradoxa*). Parsimony analysis yielded 864 shortest trees of 807 steps (CI = 0.445; RI = 0.788). The *trn*L intron alignment included 58 sequences and 664 characters, of which 89 were informative in the P analysis; the length of the *trn*L intron sequences within the ingroup varied from 485 bp (*N. atracuminatum*) to 573 bp (*B. mucronata*). The *trn*L intron dataset resulted in 6,972 most parsimonious trees with a length of 171 steps (CI = 0.667; RI = 0.922). The strict consensus trees from individual ITS and *trn*L intron analyses are found in the supplement (Fig. S1, S2, see online supplemental material, which is available from the article's Taylor & Francis Online page at http://dx.doi.org/10.1080/14772000.2018.1442885). For each of both markers, BI, ML, and P recovered similar topologies, showing similar clades with high confidence values. Hence, we present the best ML trees from ITS (Fig. 1) and *trn*L intron (Fig. 2) data.

The concatenated dataset (ITS+*trn*L intron) included 61 sequences and consisted of 1,253 characters, of which 308 are parsimony-informative. Parsimony analysis resulted in 198 optimum trees of 1,019 steps (CI = 0.464; RI = 0.808); the strict consensus tree is shown in Fig. S3. The best ML tree from the combined analysis is illustrated in Fig. 3. Similar to results of both ITS and *trn*L intron individual partitions, the topologies obtained for the combined ITS+ *trn*L intron dataset by P, IB, and ML analyses were highly congruent.

In all analyses conducted for individual (ITS or *trn*L intron) and combined (ITS+*trn*L intron) datasets, the genus *Bulbostylis* is paraphyletic as *Nemum atracuminatum* is embedded within it. The '*Bulbostylis-Nemum* clade' is sister to a clade including all other Abildgaardieae species: the '*Abildgaardia-Crosslandia-Fimbristylis* clade' (Figs 1–3 and S1–3, see supplemental material online).

In all three ML hypotheses, the *Bulbostylis–Nemum* clade is well-supported and includes a derived clade that comprises most American species of *Bulbostylis* (hereinafter referred to as 'American clade'; Figs 1–3). However, the three hypotheses show differences in the order of divergence of basal species, as well as in the composition and support of subclades. Other minor groups are recognized: a well-supported subclade is formed by *Bulbostylis conifera* and *B. paradoxa*, in the ITS and combined analyses, where both species are included (Figs 2 and 3); a subclade is composed by *B. funckii*, *B. hispidula*, and *B. pseudoperennis* (in the ITS and combined analyses, Figs 1 and 3), or only by *B. hispidula* and *B. pseudoperennis* (in the *trn*L intron analysis, where *B. funckii* is not included, Fig. 2), forming highly supported clades, respectively.

Incongruences amongst the trees from the individual and combined datasets are related to the location of: (1) *B. macra*, *B. pusilla*, and *B. scleropus*, which form a polytomy within the American clade (*trn*L intron tree; Fig. 2), or group together in one highly supported clade but not related to the American clade (ITS and combined trees; Figs 1 and 3); and (2) *B. junciformis*, *B. scabricaulis*, *B. sphaerocephala*, and *B. stenocarpa*, which form a moderately to highly supported clade outside the American clade (*trn*L intron tree; Fig. 2), or alternatively form a polytomy (ITS tree; Fig. 1), or a moderately to highly supported clade (combined tree; Fig. 3), within the American clade.

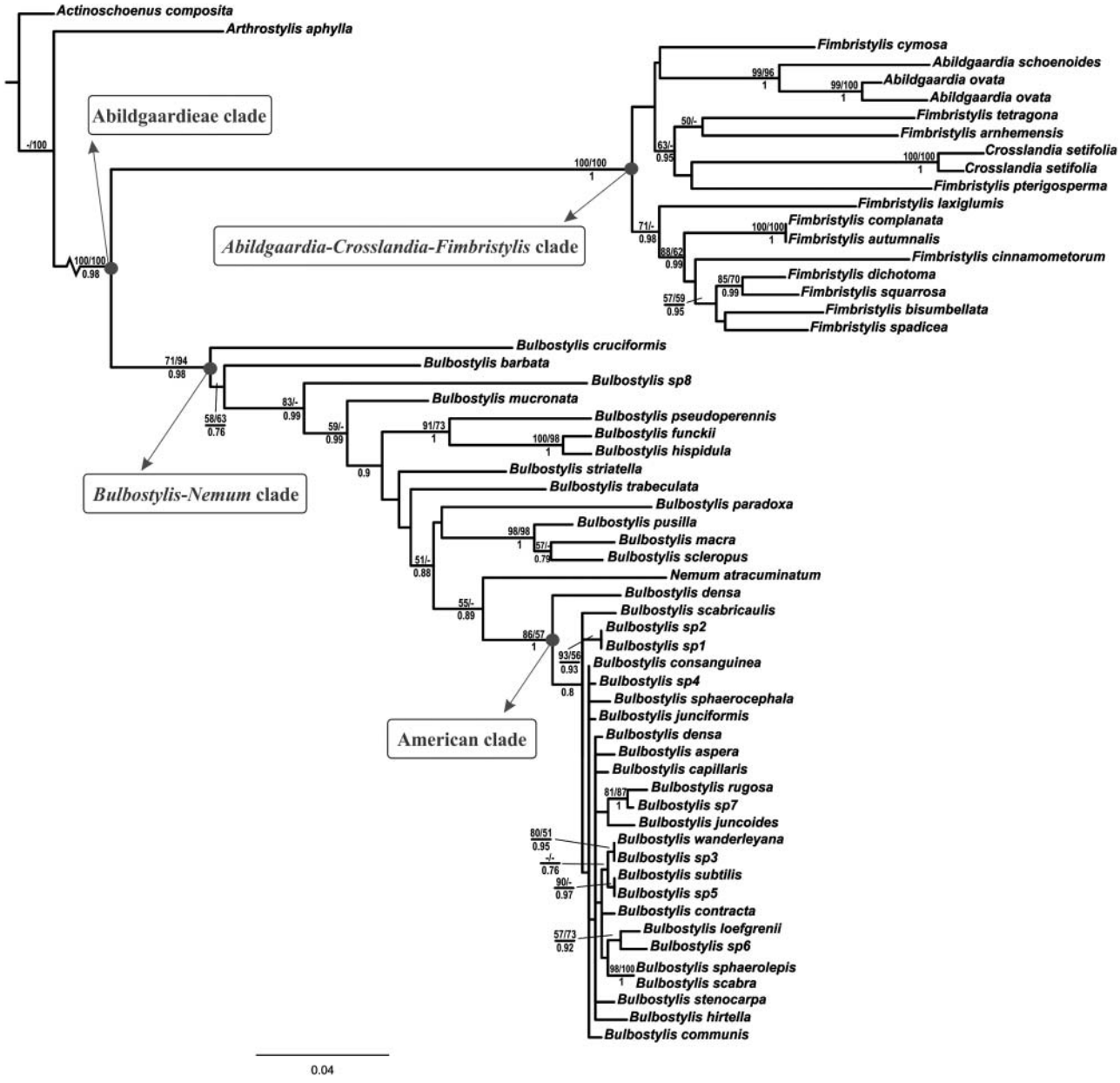

Fig. 1. Maximum likelihood tree inferred from analysis of nuclear ITS sequences. Numbers above branches represent bootstrap values from maximum likelihood/parsimony respectively; numbers below branches are Bayesian posterior probabilities.

The American clade in the ITS and combined ITS+*trn*L intron trees consist of all American species: *Bulbostylis aspera*, *B. capillaris*, *B. communis*, *B. consanguinea*, *B. contracta*, *B. hirtella*, *B. junciformis*, *B. juncoides*, *B. loefgrenii*, *B. rugosa*, *B. scabra*, *B. sphaerocephala*, *B. sphaerolepis*, *B. stenocarpa*, *B. subtilis*, *B. wanderleyana*, all unpublished species of *Bulbostylis* (*sp1–sp8*) and two species not found in America: *B. densa* and *B. scabricaulis* (Figs 1 and 3). Additionally, the American clade in the *trn*L intron tree is similar to that observed in the ITS and ITS+*trn*L intron trees, although not including *B. junciformis*, *B. scabricaulis*, *B. sphaerocephala* nor *B. stenocarpa* (*B. contracta* is not represented by a *trn*L intron sequence), and contains three additional African species: *B. pusilla*, *B. scleropus*, and *B. macra* (Fig. 2).

Within the American clade, relationships amongst species are barely resolved. For this clade, both the ITS and combined ITS+*trn*L intron trees show weakly to moderately supported monophyletic groups (*B. sp6*+*B. loefgrenii*, *B. sp1*+*B. sp2*, *B. rugosa*+*B. sp7*, *B. scabra*+*B. sphaerolepis*, *B. wanderleyana*+*B. sp3*, *B. sp5*+*B.*

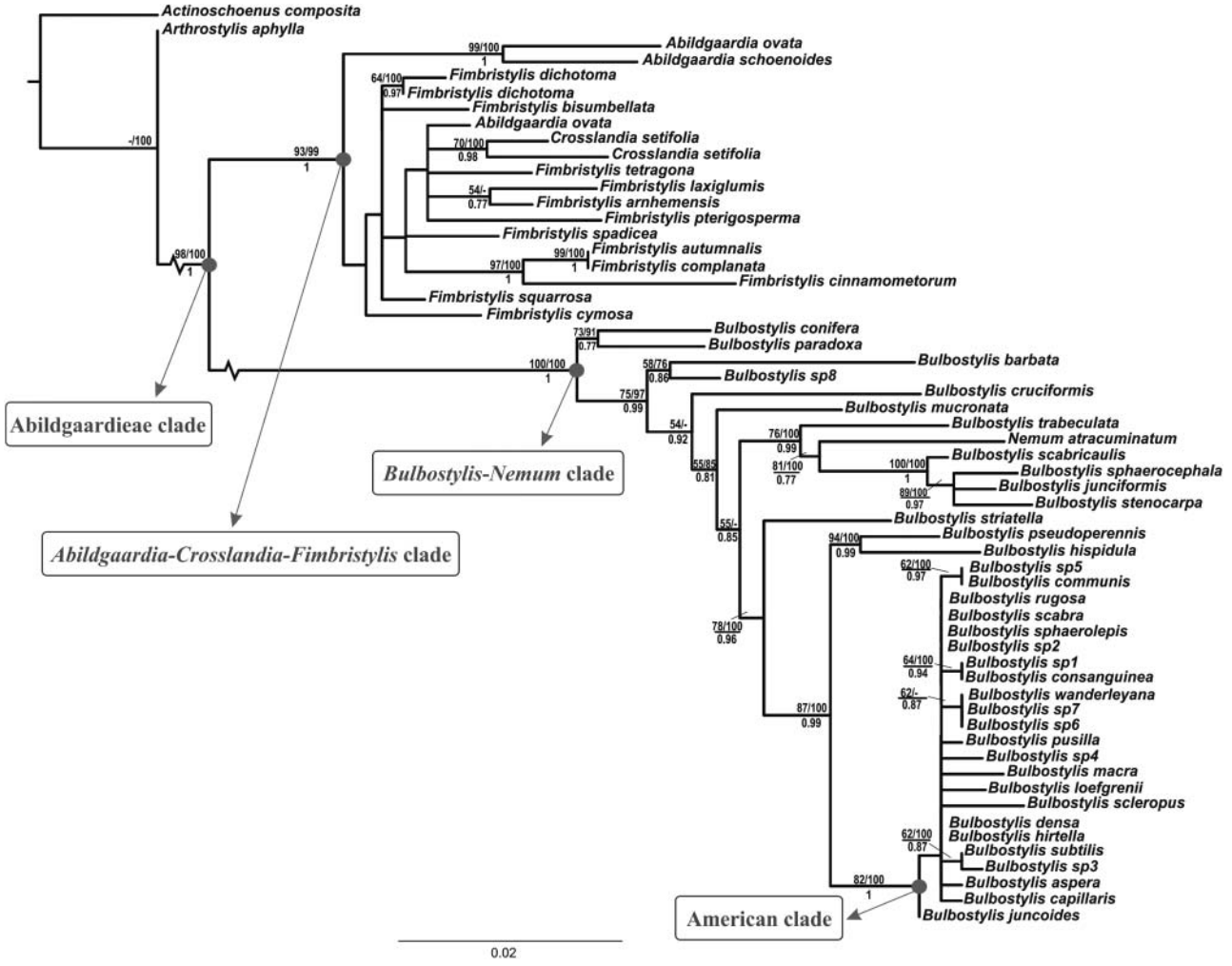

Fig. 2. Maximum likelihood tree inferred from analysis of plastid *trn*L intron sequences. Numbers above branches represent bootstrap values from maximum likelihood/parsimony respectively; numbers below branches are Bayesian posterior probabilities.

subtilis; Figs 1 and 3), which do not match the weakly to moderately supported groups retrieved for the American clade in the *trn*L intron ML tree (*B. subtilis*+*B. sp3*, *B. sp5*+*B. communis*, *B. sp1*+*B.consanguinea*, *B. wanderleyana*+*B. sp7*+*B. sp6*; Fig. 2).

The ancestral state reconstruction of the native distribution (Fig. 4) suggests an African ancestor of the *Bulbostylis–Nemum* complex based both on P and BI (lnBF = 2.83). Additionally, the introduction of species of *Bulbostylis* to America is reconstructed here in three independent events; with the American clade being the most diversified after colonization. If *B. densa* and *B. scabricaulis* are included in the American clade, both species represent posterior reintroduction to Africa probably by long dispersal events. The introduction of species to Asia and Oceania is reconstructed as several independent events.

Discussion

This work is the most comprehensive phylogenetic study of *Bulbostylis* to date, including species from different continents. A previous work (Ghamkhar et al., 2007) that examined *Bulbostylis*, only incorporated four species, three of them Australian, and one native to Africa. Our phylogenetic results show a paraphyletic *Bulbostylis*, with *Nemum atracuminatum* nested within the genus. These findings, along with those results obtained by Muasya et al. (1998, 2009) and Simpson et al. (2007), who observed a close relationship between *B. atrosanguinea*, *B. hispidula*, and *N. spadiceum*, are strong signals to consider *Bulbostylis* and *Nemum* as part of a natural group.

The presence in both genera of woolly hairs at the mouth of leaf sheaths (Goetghebeur, 1998; Goetghebeur & Coudijzer, 1984; Larridon, Reynders, & Goetghebeur, 2008) and of a wide turbinate embryo with the embryonic

Fig. 3. Maximum likelihood tree inferred from analysis of combined *trn*L intron and ITS sequences. Numbers above branches are bootstrap values from maximum likelihood/parsimony respectively; numbers below branches are Bayesian posterior probabilities.

axis totally recurved and the radicle and plumule in basal position (= *Bulbostylis*-type embryo; Van der Veken, 1965), represent probable synapomorphies of the *Bulbostylis–Nemum* clade. Unlike *Bulbostylis* and *Nemum*, in *Abildgaardia*, *Crosslandia*, and *Fimbristylis* the leaf sheaths orifice is glabrous (Goetghebeur, 1998; Goetghebeur & Coudijzer, 1984), and the embryo is about turbinate with the radicle in lateral position and the plumule in basal position (= *Fimbristylis*-type embryo; Van der Veken, 1965), or it is similar to those of *Bulbostylis*-type but with a great development of the second leaf and with a third additional leaf (= *Abildgaardia*-type embryo; Van der Veken, 1965).

Within *Bulbostylis* none of the infrageneric groups suggested by López (2012) and by López and Gonzalez (2017) based on micromorphology of achene surface was retrieved as monophyletic in our molecular phylogenetic study (Fig. 4; Table 1). López (2012) and López and Gonzalez (2017) proposed three large groups of species: (a) fruit exocarp without silicophytoliths (group 1; Figs 5.1, 5.2); (b) fruit exocarp with silicophytoliths of undefined shape, which occupy most of the cell (group 2; Figs 5.3, 5.4); and (c) fruit exocarp with silicophytoliths of defined shape, located at the centre of the cell or excentrically (group 3; Figs 5.5, 5.6). Our results show the limited value of the fruit micromorphology to establish infrageneric groups in *Bulbostylis*.

Otherwise, the current phylogenetic context reveals the homoplasic condition of the diagnostic character 'style-base permanence on the ripe fruit'. The style base duration has been commonly used to differentiate *Bulbostylis* from *Abildgaardia* and *Fimbristylis*. The style base is a persistent structure in most species of *Bulbostylis* and a deciduous one in *Abildgaardia* and *Fimbristylis*. However, this

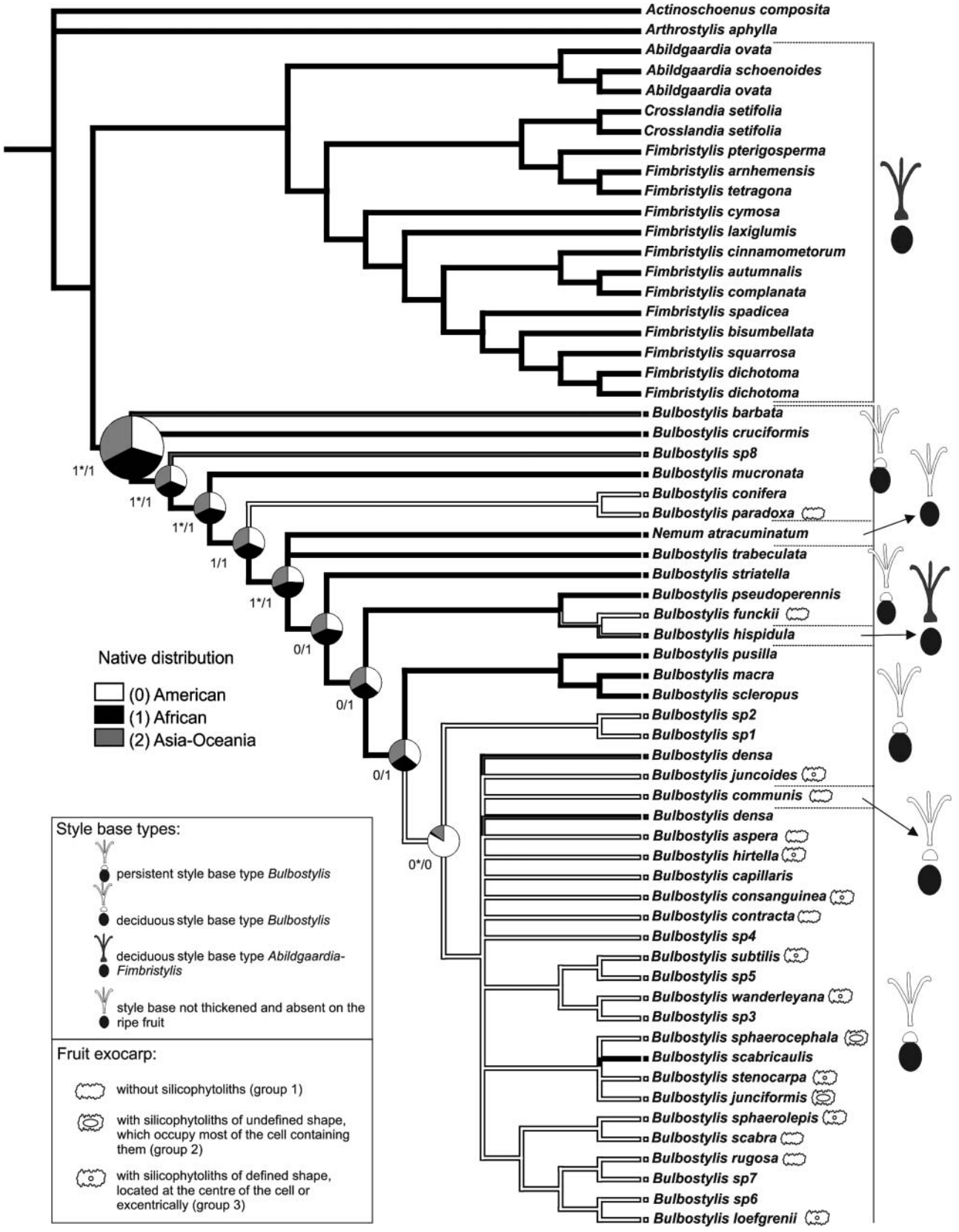

Fig. 4. Reconstructed ancestral character states of native distribution on the Bayesian MCMC majority rule consensus tree (15,002 trees), and mapping of some morphological characters discussed in the text. Branch shading indicates parsimony reconstruction. Pie charts indicate Bayesian ancestral characters posterior probabilities at selected nodes. First number below charts indicates the state with the highest likelihood based on the Bayes factor (the asterisks imply Bayes factor > 2). The second numbers specify the state indicated by the parsimony reconstruction.

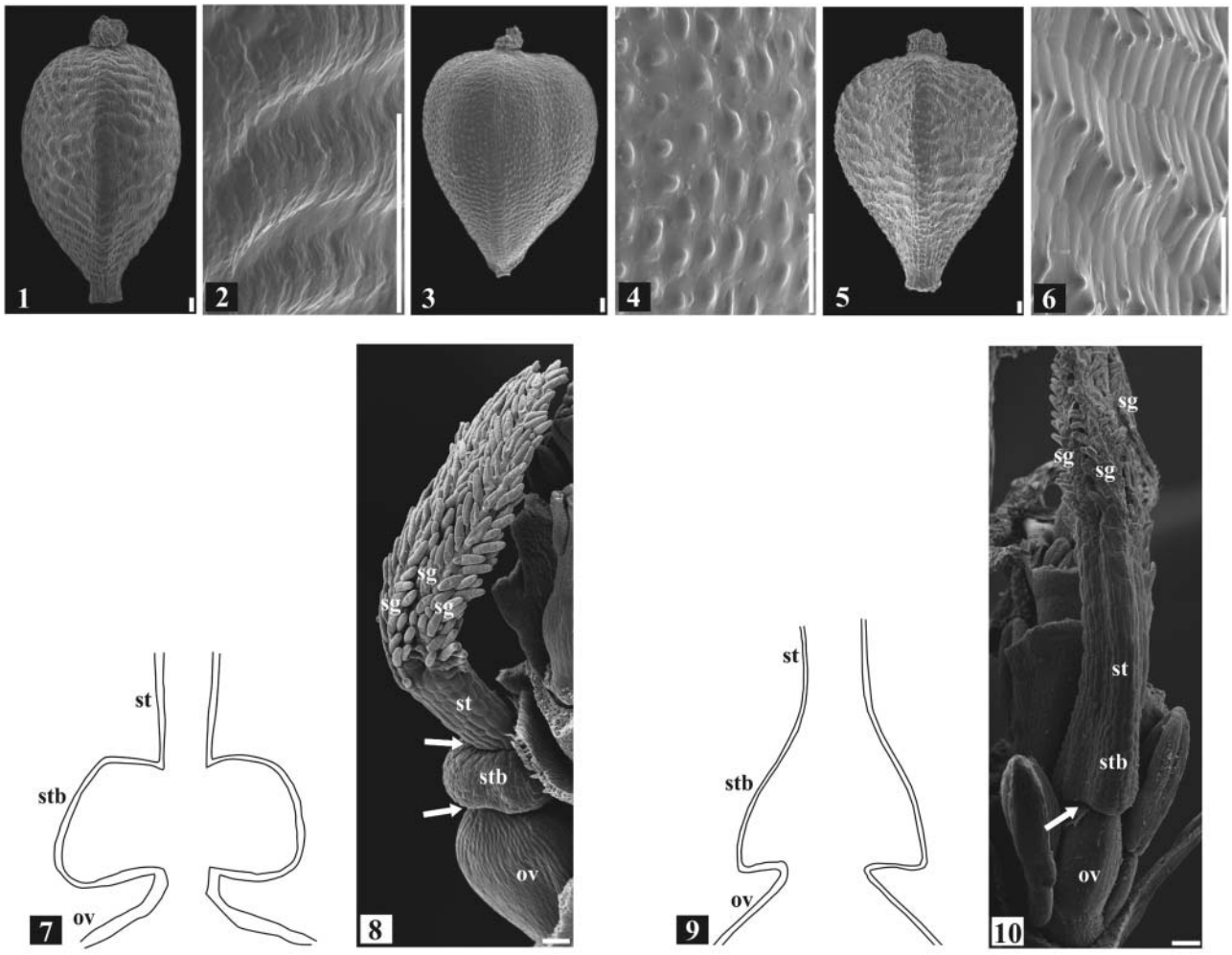

Fig. 5. 1–5.10. Character states of fruit micromorphology in South American species of *Bulbostylis* according to López (2012) and López and Gonzalez (2017), and style base structure in *Abildgaardia*, *Bulbostylis*, and *Fimbristylis* according to Reutemann et al. (2012): **5.1–5.2**, *Bulbostylis aspera*, fruit exocarp without silicophytoliths (group 1), **5.3–5.4**- *Bulbostylis sphaerocephala*, fruit exocarp with silicophytoliths of undefined shape, which occupy most of the cell containing them (group 2), **5.5-5.6**, *Bulbostylis juncoides*, fruit exocarp with silicophytoliths of defined shape, located at the centre of the cell or eccentrically (group 3), **5.7–5.8**, *Bulbostylis juncoides*, style base type *Bulbostylis*, **5.9–5.10**, *Fimbristylis complanata*, style base type *Abildgaardia-Fimbristylis*. References: ov, ovary wall; sg, stigma; st, style; stb, style base. Scale bars = 50 μm.

character is not decisive, since there are species of *Bulbostylis* without a persistent style base (e.g., *B. communis*, *B. hispidula*, *B. sellowiana*). Moreover, the species of *Bulbostylis* with a deciduous style base included in the analyses, *B. communis* and *B. hispidula*, do not form a clade in any of the analyses. Although *Nemum atracuminatum* also presents a deciduous style base, this species is apart from *B. communis* or *B. hispidula*.

The deciduous style base of *B. communis* and *B. hispidula* belong to different morphological types according to Reutemann, Vegetti, and Pozner (2012). These authors describe two types of style base: (1) *Bulbostylis* type, which has two joints: one at the ovary apex and another one at the distal end of the style base (Figs 5.7, 5.8); and (2) *Abildgaardia–Fimbristylis* type, with a single joint with the ovary apex (Figs 5.9, 5.10). *Bulbostylis communis* has a *Bulbostylis*-type style base (which is typical of *Bulbostylis* species), whereas *B. hispidula* shows an *Abildgaardia-Fimbristylis*-type (which is typical of *Abildgaardia* and *Fimbristylis* species). In *B. hispidula*, the presence of an *Abildgaardia–Fimbristylis*-type style base has led other authors to consider this taxon as *Fimbristylis*; however, the presence of a *Bulbostylis* embryo type and woolly hairs at the apex of the leaf sheath emphasize its association to *Bulbostylis*. Our phylogenetic analyses support the inclusion of *B. hispidula* within *Bulbostylis*, as well as the concept of independent acquisition of an *Abildgaardia–Fimbristylis*-type style base (Fig. 4).

All three ML trees obtained in this work (Figs 1–3) show very short branch lengths for most of the species of the

American clade, compared with the rest of the species. Based on this fact, and considering the high morphological similarity of species in the American clade, we might assume a rapid diversification of this clade, which might result from the acquisition of some key structural innovation possibly accountable for a burst of speciation. A thorough morphological study covering broadly distributed species of *Bulbostylis* might identify synapomorphies for the American clade, and thus supporting or refuting this assumption. Incorporation of additional markers might also contribute towards resolving this polytomy; moreover, new technologies based on next-generation sequencing might better deal with difficulties posed by polyploidy, hybridization, and recent radiations (Harrison & Kidner, 2011); Larridon et al. (2013) mention this issue regarding the polytomy obtained for the majority of C_4 *Cyperus* spp.

Bulbostyllis conifera and *B. paradoxa* appear as a moderately to highly supported monophyletic group in all analyses. Both species, along with *B. funckii*, are the only species native to America that fall outside the American clade in all analyses. These three species show a contrasting morphology not shared with the other American species, such as the presence of a caudex in *B. paradoxa*, and unispicate inflorescences in *B. conifera* and *B. funckii*.

Based on the reconstruction pattern of the native distribution presented in this work, the ancestor of *Bulbostylis* plus *Nemum atracuminatum* is likely to be African. It is necessary to obtain sequences of the monotypic genus *Nelmesia* to test its likely closeness to *Bulbostylis* and *Nemum*, so far assumed on the basis both of its African distribution (Goetghebeur, 1998) and its *Bulbostylis*-type embryo (Van der Veken, 1965).

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Supplemental data

Supplemental data for this article can be accessed at: https://doi.org/10.1080/14772000.2018.1442885

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