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EVOLUTION AND DEVELOPMENT OF THE SPIKELET AND FLOWER OF *RHYNCHOSPORA* (CYPERACEAE)

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Premise of research. *Rhynchospora*, one of the biggest genera of sedges, displays several spikelet and flower modifications and is one of a few genera where the pollination strategy changes from wind to insect pollination; however, the mechanisms that have promoted such diversity in the genus are unknown. We have performed a comparative study of spikelet and flower development of *Rhynchospora* from an evolutionary perspective to get insight into the bases of spikelet and flower evolution. Particularly, we investigate correlations between the change from wind to insect pollination that occur in the genus and the spikelet and flower traits.

Methodology. The spikelet and flower development of nine species of *Rhynchospora* were studied using SEM images. Parsimony, maximum likelihood, and Bayesian reconstruction analyses were conducted to evaluate the evolutionary history of flower and spikelet developmental programs.

Pivotal results. We identified seven morphological and developmental traits that vary among *Rhynchospora* species; ancestral character reconstructions show that the spikelet and flower morphology and their developmental pathways support the diversification of *Rhynchospora* lineages.

Conclusions. Our results suggest that the evolution of spikelet and flower developmental programs in *Rhynchospora* may be related to the evolution of its pollination type and mating system. The evolution toward a bisexual spikelet and flowers in which the androecium develops faster than the gynoecium may represent important steps for the transition from a wind- to insect-pollination system. In addition, we found new traits of spikelets and flowers that may help to distinguish natural groups within *Rhynchospora*.

Keywords: Rhynchospora, flower, spikelet, development, evolution, Cyperaceae.

Online enhancements: appendixes.

Introduction

Angiosperms display a remarkable diversity of flowers. In the past 2 decades, botanists have started to pay particular attention to how the reproductive structures of angiosperms are developed (Endress 1990; Endress and Doyle 2009; Hileman and Irish 2009; Soltis et al. 2009; Crane et al. 2010). The study of the reproductive structures of flowering plants from early developmental stages to maturity has become a valuable tool not only to asses homology questions on morphological evolution but also to understand functional processes (Marazzi et al. 2007; Reinheimer et al. 2010). Moreover, the use of developmental timing events has gained importance in phylogenetics (Laurin and Germain 2011); in this sense, ontogenetic and phylogenetic approaches have been linked in order to assess the evolution of developmental patterns of flowers, spikelets, and inflorescences in angiosperms (Doust and Kellogg 2002; Doust and Drinnan 2004; Kellogg et al. 2004; Bess et al. 2005; Jabbour et al. 2008, 2009; Feng et al. 2011).

Cyperaceae (sedges) are one of the most species-rich families of flowering plants. They are currently divided into two subfamilies, Cyperoideae and Mapanioideae (Muasya et al. 2009). In Cyperoideae, the spikelet may include only one flower (*Ascolepis*; Goetghebeur 1998) or even more than 10 flowers (*Scleria*; Ahumada 2007). The inflorescence of Cyperoideae has been considered a panicle of spikelets (Goetghebeur 1998), where the spikelets are functionally the reproductive unit of the inflorescence. Flowers in Cyperoideae may be bisexual or unisexual (male or female). A typical Cyperoideae bisexual flower consists of two perianth whorls (exceptions occur in tribes Cypereae and Cariceae, the flowers of which lack a perianth; Goetghebeur 1998), an androecium, and a central gynoecium (Bruhl 1991). These whorls are trimerous, like the typical monocot flower, although there are variations that can

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be understood on the basis of this general model (Vrijdaghs et al. 2009). Flowers in Cyperoideae may vary in the morphology and number of perianth parts when present, the number of stigmatic branches, and the number of stamens (Goetghebeur 1986, 1998; Vrijdaghs et al. 2004, 2005*a*, 2005*b*, 2006, 2009); such modifications could alter the common radial symmetry of the flowers to a bilateral symmetry of each whorl (Prenner et al. 2010).

Subfamily Cyperoideae has ~90 genera, the largest being *Carex* (more than 1828 species) and *Cyperus* (more than 702 species; Govaerts et al. 2013); *Rhynchospora* Vahl, with ~354 species, is the third-largest genus in subfamily Cyperoideae and is mainly distributed in the American savannas (Koyama 1972; Strong 2006; Thomas et al. 2009). The current classification of the genus follows Kükenthal (1949, 1950, 1951, 1952). Kükenthal recognized two subgenera based on the division of the style: a shallowly divided style for *Rhynchospora* subgenus *Haplostyleae* and a deeply divided style for *Rhynchospora* subgenus *Diplostyleae* (today's subgenus *Rhynchospora*). Other characters commonly used to circumscribe the species of *Rhynchospora* were the inflorescence type, the presence/absence of perianth, and the shape and size of the fruit (Kükenthal 1949, 1950, 1951, 1952; Koyama 1972; Thomas 1992; Strong 2006). Recently, in a preliminary analysis of the Rhynchosporeae based on molecular data (*trn*L intron and *trn*L-F intergenic spacers), Kükenthal's infrageneric division based on the morphology of the style was not supported by the phylogeny (Thomas et al. 2009). According to these results, *Rhynchospora* may be paraphyletic relative to the genus *Pleurostachys* (member of the Rhynchosporeae tribe), and the subgenus *Haplostyleae* (Kükenthal 1949, 1950, 1951, 1952) appears to be divided into several smaller clades instead of being a natural group. Consequently, it was suggested that the clades of *Rhynchospora* should be recognized as several monophyletic genera (Thomas et al. 2009). Nevertheless, until now, no nomenclatural change has been made for the genus *Rhynchospora*, and therefore the classification of Kükenthal (1949, 1950, 1951, 1952) is still used.

In terms of reproductive structures, *Rhynchospora* shows a large variation in its inflorescence forms, spikelet and flower morphology, and pollination system. The inflorescence of *Rhynchospora* may be paniculate, capitate, or even reduced to a single spikelet (Kükenthal 1949, 1950, 1951, 1952; Koyama 1972; Thomas 1992; Guaglianone 2001; Strong 2006; Lucero and Vegetti 2012). Each spikelet has two or three basal sterile glumes and several distal fertile glumes. Two distinct spikelet types have been described for the genus: (1) a spikelet that bears exclusively bisexual flowers and (2) a spikelet that consists of a proximal bisexual flower and several distal male flowers (Kükenthal 1949, 1950, 1951, 1952; Koyama 1972; Thomas 1992; Guaglianone 2001; Strong 2006). A typical flower of *Rhynchospora* has a perianth with an outer and an inner whorl of three tepals each (some species may have flowers without a perianth), an androecium consisting of one to three stamens, and a gynoecium with two stigmatic branches (Kükenthal 1949, 1950, 1951, 1952; Koyama 1972; Thomas 1992; Guaglianone 2001; Strong 2006). In addition, the genus is characterized by a persistent style base in the fruit (Kükenthal 1949, 1950, 1951, 1952; Koyama 1972; Thomas 1992; Guaglianone 2001; Strong 2006).

In regard to the pollination mode, in general, the species of the genus *Rhynchospora* are pollinated by wind or sometimes by insects (Thomas 1984). In this sense, the genus *Rhynchospora* is one of a few genera of Cyperaceae with entomophilous

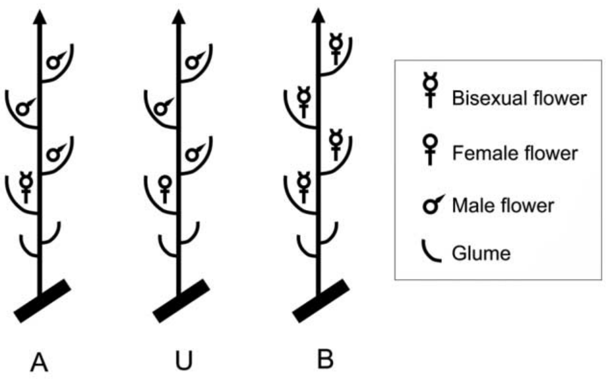

Fig. 1 Simplified schemes of *Rhynchospora* spikelet types based on differences in sexual systems. All spikelets have two or three basal sterile glumes. Andromonoecious spikelets (A) consist of a proximal bisexual flower and three to five distal male flowers. Unisexual spikelets (U) have a proximal female flower and three distal male flowers. Bisexual spikelets (B) bear five to six bisexual flowers exclusively.

Table 1

Morphological and Developmental Characters of Spikelets and Flowers of *Rhynchospora*

Species	No. basal sterile glumes	Sexual system	No. stamens at the bisexual flower	Perianth	No. stigmatic branches	Style length	Relative rates of Rhynchospora androecium/gynoecium elongation
R. brittonii	2	B	1	Present	2	Short	Type 3
R. consanguinea	2	A	2	Present	2	Long	Type 2
R. corymbosa	3	A	3	Present	2	Long	Type 1
R. pilosa	2	A	2	Absent	1	Long	Type 2
R. riedeliana	2	A	2	Absent	2	Long	Type 2
R. scutellata	3	A	3	Present	2	Long	Type 2
R. setigera	3	B	3	Absent	2	Short	Type 4
R. tenuis	3	B	3	Absent	2	Short	Type 4
R. terminalis	2	U	NA	Present	2	Long	NA

Note. A, andromoecy; B, bisexual; U, unisexual; NA, not applicable. See text for further explanation of the four relative rates of androecium/gynoecium elongation.

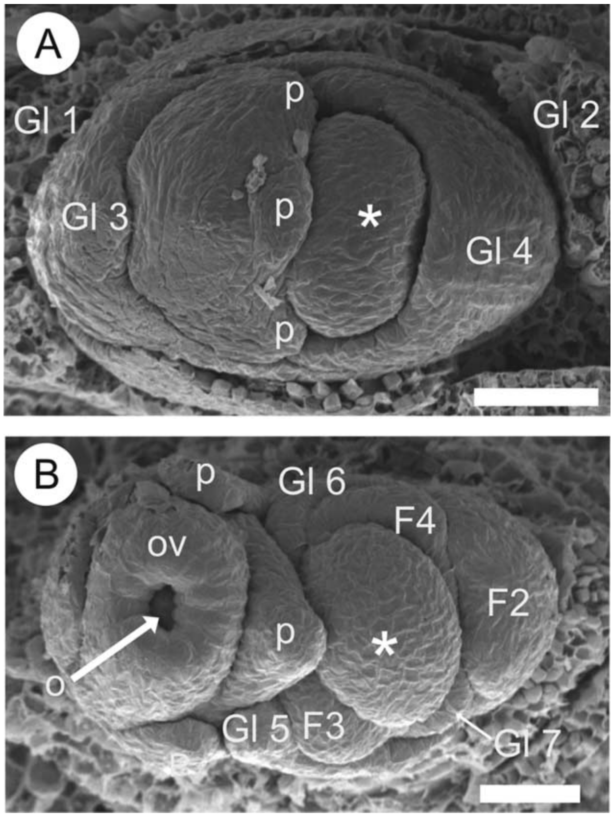

Fig. 2 Spikelet development in *Rhynchospora terminalis* as an example of spikelet development in *Rhynchospora*. *A*, Two basal sterile glumes (Gl 1 and Gl 2), with distichous arrangement, are the first organs to be initiated. The floral meristem is emerging in the axil of the third glume (Gl 3), which arises opposite the fourth glume (Gl 4). The rachilla apex (indicated by an asterisk) is visible between the floral meristem and Gl 4. *B*, When the central ovule and the ovary wall of the most proximal flower start to differentiate, two other flower primordia begin their development at the axil of the fifth (Gl 5) and sixth (Gl 6) glumes. Fertile glumes are spirally located (cf. the position of Gl 4 to Gl 7). F = floral meristem (F2, second flower; F3, third flower; F4, fourth flower), Gl = glume, o = ovule, ov = ovary wall, pi = inner perianth part, po = outer perianth part. Scale bars: *A*, *B* = 50 μm.

species derived from anemophilous species; other examples were recorded in *Cyperus*, *Carex*, *Ficinia*, and in some Mapanioideae (Wragg and Johnson 2011), given that the ancestral condition of the family is wind pollination (Thomas 1984; Goetghebeur 1998; Givnish et al. 2010; Wragg and Johnson 2011). Species that were recorded to be visited by insects occur in *Rhynchospora* section *Dichromena*, whose capitate inflorescences are visited by flour beetles, syrphid flies, and bees (Thomas 1984); species of *Rhynchospora* section *Dichromena* have white glumes and sticky pollen, which are traits linked to entomophilous pollination (Thomas 1984). There are no reports of other members of genus *Rhynchospora* s.s. being insect pollinated. Recently, Wragg and Johnson (2011) found that pollen motility, floral scents, and inflorescence color are traits correlated with the transition from wind pollination to insect pollination in *Cyperus*. In general, in angiosperms, it is well documented that floral architecture; sexual system, stamen, and stigma characteristics; and the protandrous or protogynous condition of bisexual flowers are also traits (hereafter meaning the state of a given character) associated with changes in the pollination mode (Sargent and Otto 2004; Friedman and Barrett 2009; Givnish et al. 2010); however, the relationship between these traits and the evolution of an insect-pollination mode has not been examined in Cyperaceae. Given the variability of reproductive structures and the pollination modes mentioned earlier, genus *Rhynchospora* s.l. represents an interesting opportunity to look for correlations between the evolution of the spikelet, floral traits, and pollination mode.

Rhynchospora species display several spikelet and flower modifications; however, the mechanisms that have produced such diversity in the genus are unknown, as are their homologies. Furthermore, the correlation between the evolution of spikelet and floral developmental traits of *Rhynchospora* and the transition toward an insect-pollination mode never was explored. To fill these gaps, we determined to (1) investigate the mechanisms that have promoted the morphological diversity of flowers and spikelets in the genus, (2) test the homology of the characters found in the genus, and (3) investigate the relationship between the floral and spikelet traits and pollination mode of *Rhynchospora* species. In order to achieve these goals, we undertook a comparative study of spikelet and flower development using SEM and studied their evolution in a molecular phylogenetic context.

Material and Methods

Developmental Studies (SEM Images)

Nine species were selected depending on material availability, including at least one representative of all the major clades of the genus *Rhynchospora*, according to the most recent phylogeny (Thomas et al. 2009). Four of the species studied here were courtesy of Shirley Martins (Universidade Estadual Paulista, São Paulo, Brazil; app. A; see also app. B, available online). q2 Inflorescences at different stages of development were collected from the field, fixed in FAA (formaldehyde–ethanol–acetic acid–water, 10 : 50 : 5 : 35 v/v) solution for 24 h, and then transferred to 70% ethanol.

Spikelets were dissected under a Nikon SMZ-10 stereoscopic microscope to remove glumes and expose the floral organs. The dissected spikelets were dehydrated in an increasing series of ethanol, up to absolute ethanol. The material was then transferred to pure acetone for desiccation using CO_2 in a critical-point dryer (Emitech K850). Mounted samples were coated with gold/palladium using a Thermo VG Scientific Polaron SC7620 sputter-coater (Zürich) or with gold using a Model 3 Polaron 91000 sputter-coater (Pelco) and then photographed using a Philips XL30 Series (Eindhoven, the Netherlands) scanning electron microscope at the Museo Argentino de Ciencias Naturales Bernardino Rivadavia in Buenos Aires and an EVO40 VP scanning electron microscope (Cambridge) at the Centro Científico Tecnológico Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) Bahía Blanca in Buenos Aires, respectively. q3

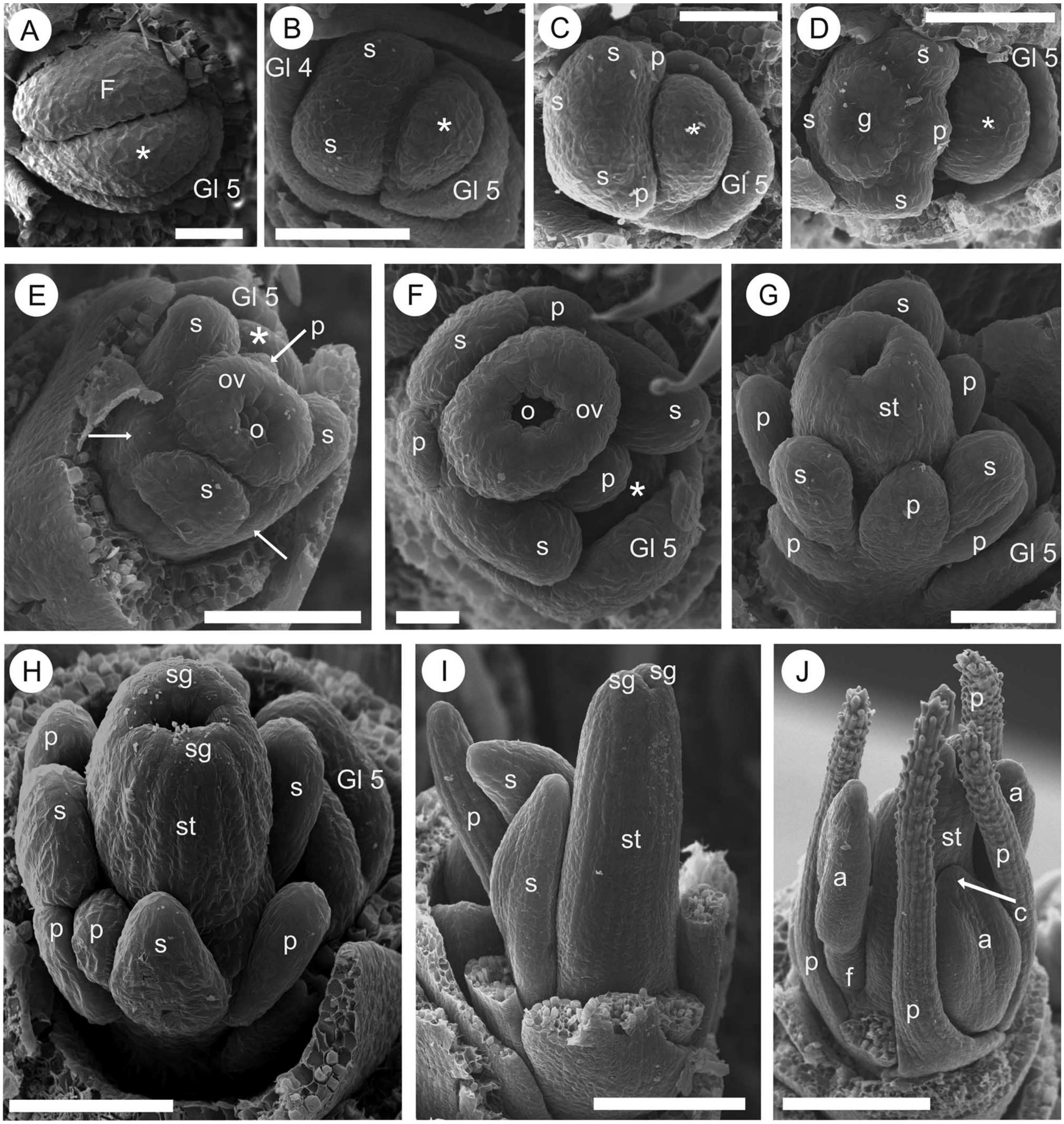

Fig. 3 Floral development in *Rhynchospora corymbosa* as an example of floral development in *Rhynchospora*. Three basal sterile glumes were removed. *A*, The initiation of a flower begins with the development of a dome-shaped meristem that arises at the axil of a fertile glume. *B*, Two lateral stamen primordia are the first floral organs to initiate. *C*, Then, a third stamen primordium and the outer whorl of perianth primordia are initiated; outer perianth primordia develop opposite and below the stamens. *D*, Later, the gynoecium primordium appears simultaneously with the inner perianth (arrows); inner perianth primordia develop between stamens. *E*, Then, the ovule and the ovary wall start to differentiate. *F*, The ovary encloses the ovule. *G*, The style elongates surpassing the stamens. *H*, Two shallow stigmas appear. *I*, The style continues growing, whereas stigmas remain brief. *J*, In advanced stages of development, the perianths became scabrid. a = anther, c = connective, f = filament, F = flower meristem, g = gynoecium, Gl = glume, o = ovule, ov = ovary wall, pi = inner perianth part, po = outer perianth part, s = stamen, st = style, sg = stigma, asterisk = rachilla apex. Scale bars: *A*, *F* = 20 μm; *B* = 30 μm; *C*–*E*, *G*, *H* = 50 μm; *I*, *J* = 100 μm.

DNA Extraction and Sequencing

Total DNA was extracted from silica-dried tissue using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions or the CTAB method (Doyle and Doyle 1987). The plastid *trn*L (UAA) intron and the intergenic spacer between the *trn*L (UAA) $3'$ exon and the *trn*F (GAA) gene (*trn*L-F region) were amplified from total DNA using the polymerase chain reaction (PCR) with universal primers *c* and *f* (Taberlet et al. 1991). PCR reactions were performed in a final volume of 25 μL containing 0.15 μL of *Taq* polymerase (Universidad Nacional de Quilmes), 2.5 μL 10 × buffer, 2.5 μL 5 mM $MgCl_2$, 1.5 μL 10 μM of each primer, 0.25 mM of each dNTP, and 0.75 μL of dimethyl sulfoxide. Amplifications were performed in a TGradient Thermocycler (Biometra, Göttingen, Germany), with an initial denaturation for 5 min at 94°C, followed by 36 cycles at 94°C for 30 s, 48°C for 1 min, 72°C for 1.5 min, and a final extension at 72°C for 10 min.

PCR products were cleaned and sequenced by Macrogen (Seoul) using the ABI PRISM BigDye Terminator Cycle Sequencing Kits with AmpliTaq DNA polymerase (Applied Biosystems, Seoul). Single-pass sequencing was performed using the same primers used for PCR reactions. The sequences were assembled and edited in BioEdit, version 7.0.9.0 (Hall 1999). Sequences were aligned using MUSCLE, version 3.8 (Edgar 2004*a*; 2004*b*), and inspected by eye. GenBank accession numbers are provided in appendixes A, B. Alignment and phylogenetic trees were submitted to TreeBASE (accession no. S13566).

Phylogenetic Analysis

The outgroup taxa were selected based on the most recent molecular phylogeny of Cyperaceae (Muasya et al. 2009). Three species were included as outgroup taxa, *Cladium mariscus*, *Scleria distans*, and *Schoenus nigricans*; their sequences were retrieved from GenBank (DQ058298, DQ058299, and AJ295814, respectively). Maximum likelihood (ML) and Bayesian (BI) analyses implemented the GTR+G model of evolution, based on tests for the best-fit model using the Akaike information criterion (AIC) executed in jModelTest 0.1.1 (Posada 2008).

ML analyses were performed using PhyML, version 3.0 (Guindon and Gascuel 2003; Guindon et al. 2010), with a BIONJ (an improved version of the neighbor-joining algorithm) tree as a starting point, using nearest neighbor interchange moves for tree searching. Nodal robustness on the ML tree was estimated by the nonparametric bootstrap (BS) with 1000 replicates.

Bayesian phylogenetic estimates were run for 6 million generations in MrBayes, version 3.1.2 (Huelsenbeck and Ronquist 2001). Four Markov chains were run simultaneously in two independent runs, starting with a random tree. Trees were sampled every 1000 generations. Convergence diagnostics for log-likelihood values were assessed visually using Tracer, version 1.5.0 (Rambaut and Drummond 2007). Burn-in for each run was determined independently by plotting $-$log likelihood against the number of generations. To calculate the Bayesian posterior probabilities (PP), trees prior to stationarity were excluded, and the remaining trees (11,981 trees) were used to generate a 50% majority rule consensus tree.

Character Evolution

In order to gain insight into the evolution of the *Rhynchospora* spikelet and flower morphology, we performed ancestral character-state reconstruction studies using the phylogeny generated in this work. We chose several qualitative characters and character states based on SEM observations; to check the suitability of the characters, we examined several specimens (apps. A, B). Although some of the characters studied here were previously used for species description and/or classification (as characters 1, 3, and 4; Kükenthal 1949, 1950, 1951, 1952; Koyama 1972; Thomas 1984, 1992; Guaglianone 2001; Strong 2006), they were never tested properly in a phylogenetic framework as they are here. The seven characters selected and their states were as follows:

1. Number of basal sterile glumes (state 0: two; state 1: three).

2. Spikelet type according to sexual systems (state 0: andromonoecious system; state 1: bisexual system; state 2: unisexual system).

3. Number of stamens in bisexual flowers (state 0: three; state 1: two). Our observations agree with previous literature, except for the number of stamens of flowers of *Rhynchospora brittonii*. Although we observed only one stamen in flowers of *R. brittonii*, we coded this character as polymorphic according to Guaglianone (1980), who found one, two, or even three stamens depending on the studied individual.

4. Presence of flowers with perianth along the spikelet (state 0: all flowers with perianth; state 1: flowers without perianth; state 2: only the proximal flower has perianth).

5. Style length (state 0: long; state 1: short). Our interpretation of the style length differs from previous treatments in that the gynoecium with short style has been considered a two-branched style (Kükenthal 1949, 1950, 1951, 1952; Koyama 1972; Thomas 1984, 1992; Guaglianone 2001; Strong 2006). In our study, we considered the style as undivided, differing only in length; the long style is almost double the size of the ovary, while the short style is shorter than the ovary.

6. Number of stigmatic branches (state 0: three; state 1: two; state 2: one at maturity).

7. Relative rates of androecium/gynoecium elongation (state 0: androecium never surpassing the gynoecium [types 1 and 2]; state 1: androecium surpassing the gynoecium sometime during development [types 3 and 4]). The four relative rates of androecium/gynoecium elongation were coded as a binary character (character 7).

Data for outgroup species were obtained from the literature (*S. nigricans*, Vrijdaghs et al. 2007; *C. mariscus*, Vrijdaghs 2006). Given that there are no published ontogenetic reports on *Scleria* species, we used the available morphological description of the adult spikelet of *S. distans* (Ahumada 2007). The inapplicable data were coded as missing data. The data set is presented in appendix C, available online.

Ancestral state reconstruction of spikelet and floral traits was performed using BI, ML, and parsimony methods. The use of different methods for character reconstruction is a common practice to avoid relying on only one method. We use the

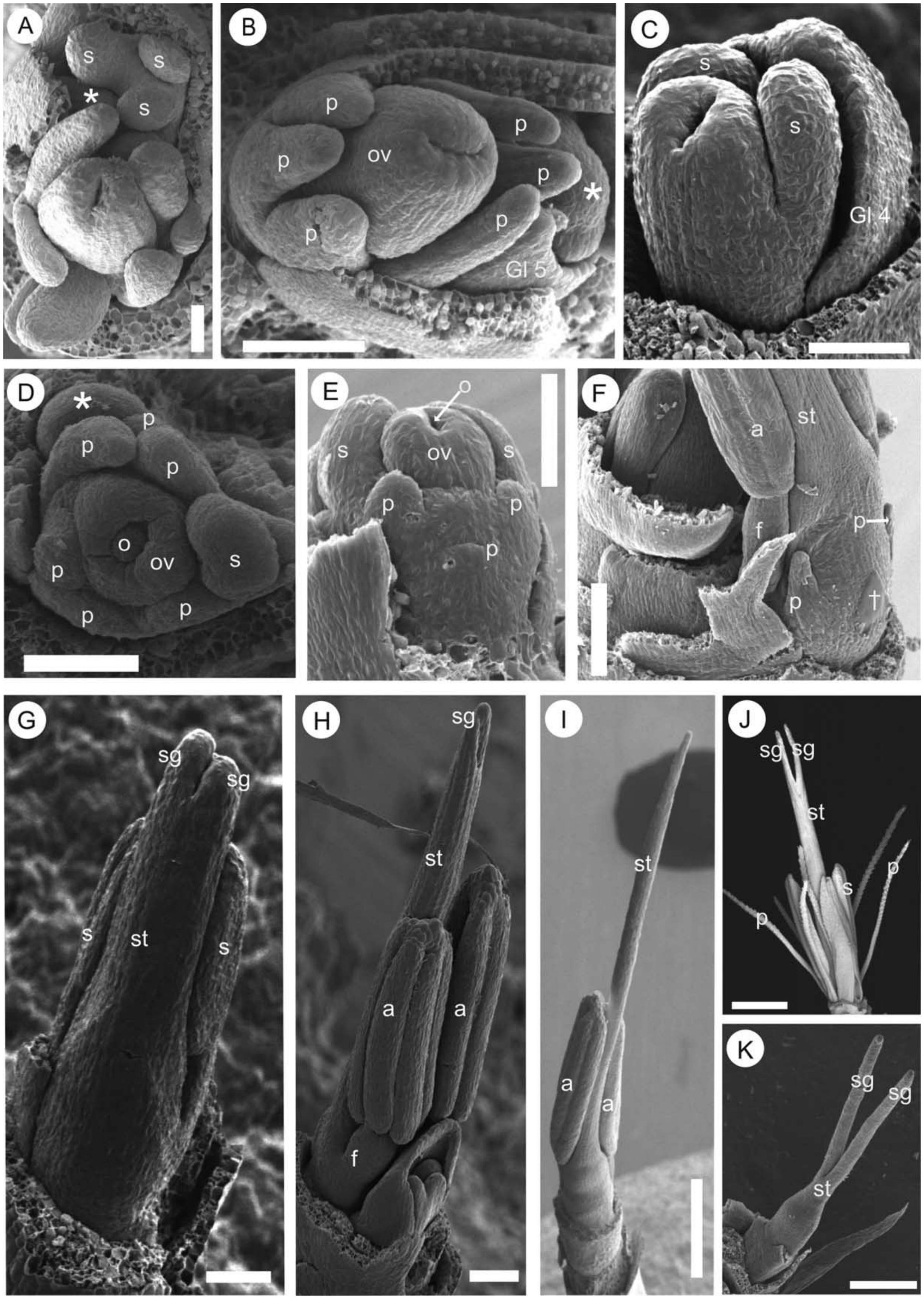

Fig. 4 Variations on the program of *Rhynchospora* flower development. Basal sterile glumes were removed. *A*, The male flower of *Rhynchospora corymbosa* develops three stamens, whereas the gynoecium and perianths never initiate. *B*, The female flower of *R. terminalis* does not develop stamens. *C*, The bisexual flower of *R. riedeliana* develops two lateral stamens, but there is no trace of the third stamen or perianths

ML tree generated by PhyML for maximum likelihood and parsimony reconstructions. For the Bayesian analysis, which is the only method that incorporates phylogenetic uncertainty into the analysis, we used BayesTraits, version 1.0 (Pagel and Meade 2006); we included the 11,981 trees obtained with MrBayes. First, we used a maximum likelihood approach to get an approximation of the rate parameters. Second, reversible-jump Markov chain Monte Carlo Bayesian analyses were performed for 10 million generations (sampling every 500 generations), setting values from 0 to 15 as priors for the exponential distribution of the rate parameters and values from 60 to 90 for the ratedev parameter. These settings allowed us to obtain acceptance rates of 20%–40%, as recommended by the authors. Tracer 1.5 (Rambautand and Drummond 2007) was used to check for adequate effective sample sizes (ESS) for all model parameters. Finally, we compared the resulting Bayes factor scores to generate an estimate for the relative support of each alternative hypothesis, using the fossil command implemented in BayesTraits, version 1.0.

Parsimony and maximum likelihood reconstructions were performed using Mesquite, version 2.75 (Maddison and Maddison 2010). The difference between these reconstruction methods is that parsimony incorporates polymorphic states, while maximum likelihood does not. We used the maximum likelihood tree generated by PhyML for this purpose. For the maximum likelihood approach, we used the Markov one-rate (Mk1) model. Characters were treated as unordered for the parsimony analysis.

Results

Scanning electron photographs that illustrate in detail the spikelet and flower development of each of the species studied q6 are provided in appendixes A, B. Given space constraints, only the most illustrative scanning electron photographs are presented q7 below. Table 1 summarizes the diversity of *Rhynchospora* spikelet and flower morphology and development found in this study.

Spikelet Morphology

In the genus *Rhynchospora*, spikelets vary in the number of sterile glumes and the sexuality of the flowers (table 1). Spikelets bear two or three basal sterile glumes depending on the species (e.g., two in *Rhynchospora terminalis* or three in *R. corymbosa*) and several distal fertile glumes. All *Rhynchospora* species examined are monoecious. We found three different spikelet types based on the sex of the flowers (fig. 1). In general, spikelets may have a proximal bisexual flower and three to five distal male flowers (andromonoecious system, A; e.g,. *R. corymbosa*). On the other hand, the spikelet of *R. terminalis* bears a proximal female flower and three distal male flowers (unisexual system, U); this spikelet type is reported here for the first time in *Rhynchospora*. Sometimes spikelets have five to six bisexual flowers (e.g., *R. tenuis*; bisexual system, B).

Spikelet and Flower Development

The spikelet consists of an indeterminate rachilla, along which glumes and flowers develop acropetally (fig. 2). In all the species examined, the basal empty glumes are distichously arranged (fig. 2*A*) and the distal fertile glumes are placed in a spirodistichous arrangement (fig. 2*B*). The initiation of a flower primordium begins with a dome-shaped meristem (fig. 3*A*). The development of a bisexual perfect flower begins with the initiation of two lateral stamen primordia (fig. 3*B*). Later, the third abaxial stamen arises simultaneously with the outer perianth primordia, which are placed opposite the stamens (fig. 3*C*). Next, the gynoecium primordium and the inner perianth whorl appear (fig. 3*D*). During ovule and ovary wall differ- q8 entiation, the rest of the perianth parts of the inner perianth whorl initiate (fig. 3*E*). Then, the ovule is covered by the ovary wall, while the stamens and the perianth continue their expansion (fig. 3*F*). Later, the style elongates (fig. 3*G*) and two short stigmas initiate (fig. 3*H*, 3*I*). Finally, the perianth parts enlarge and become scabrid at the apex (fig. 3*J*).

Variation of Floral Developmental Patterns

We found variations on the developmental pattern described above among members of *Rhynchospora*. Such differences include (1) variations in the number of developed stamens, (2) presence/absence of perianth, (3) number of stigmatic branches, (4) style length, and (5) symmetry of the flower. Male flowers develop three stamens (two lateral stamens and one adaxial and retarded stamen), whereas the gynoecium and perianth are never initiated (fig. 4*A*). In female flowers, the gynoecium and perianth are formed, but stamens are never initiated (fig. 4*B*). Bisexual flowers may develop all three stamens (e.g., *R. corymbosa*; fig. 4*A*), two lateral stamens (e.g., *R. riedeliana*; fig. 4C), or only one lateral stamen (*R. brittonii*; fig. 4*D*); we did not find rudiments of aborted stamens in those species with a reduced number of such organs. In addition, in some of the species (*R. pilosa*, *R. riedeliana*, *R. setigera*, and *R. tenuis*), bisexual flowers do not develop perianth (fig. 4*C*). In the bisexual flower of *R. consanguinea*, one of the outer perianth parts aborts, leaving a scar visible at advanced developmental stages (fig. 4*E*, 4*F*; apps. A, B).

The gynoecium of *Rhynchospora* develops two stigmatic branches, except in *R. pilosa*, which has one stigmatic branch at maturity. Developmental images showed that the stigma of

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parts. *D*, The bisexual flower of *R. brittonii* has a single stamen and a fully developed whorl of perianths. *E*, Both perianth whorls of the *R. consanguinea* flower initiate at an early stage of development. *F*, One of the outer perianth parts of the *R. consanguinea* flower arrests its development, leaving a scar (marked with a dagger) at an advanced stage of development. G, The stigma of the *R. pilosa* flower initially has two branches. *H*, One of the stigma branches of *R. pilosa* elongates faster than the other. *I*, One of the stigma branches of the *R. pilosa* flower continues the differentiation at advanced stages of development. *J*, The gynoecium of *R. scutellata* has a long style and two brief stigmatic branches. *K*, The gynoecium of *R. setigera* has a short style and two long stigmatic branches. a = anther, f = filament, Gl = glume, o = ovule, ov = ovary wall, pi = inner perianth part, po = outer perianth part, s = stamen, sg = stigma, st = style, asterisk = rachilla apex, dagger = aborted perianth part. Scale bars: *A*, *G* = 30 μm; *B*, *F* = 100 μm; C, *E* = 20 μm; *D*, *H* = 50 μm; *I*, *K* = 300 μm; *J* = 200 μm.

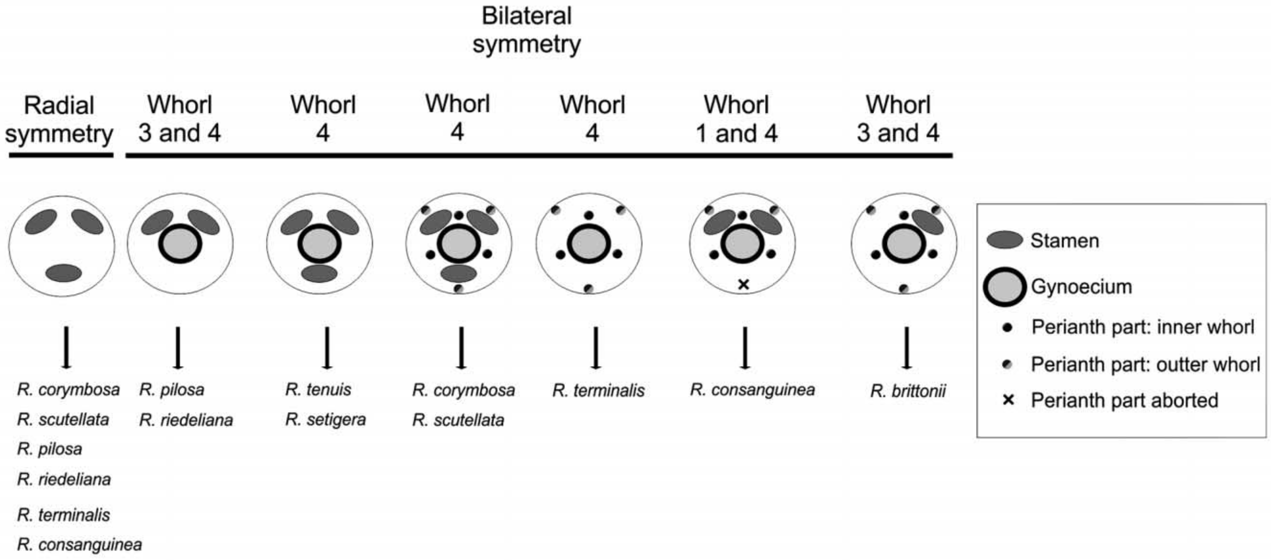

Fig. 5 Schematic representation of variability in the symmetry of *Rhynchospora* flowers. The floral whorl where the symmetry is bilateral is indicated.

R. pilosa is first divided into two branches. Later on, one of the stigmatic branches elongates, whereas the other one remains short (fig. 4*G*, 4*H*); finally, only one stigma completes its development (fig. 4*I*). The style may be long (e.g., *R. scutellata*; fig. 4*J*) or short (e.g., *R. setigera*; fig. 4*K*).

The symmetry of male flowers is radial. Bisexual and female flowers have bilateral symmetry in the fourth whorl (e.g., *R. tenuis*) given the number of stigmatic branches. In some cases, the third whorl possesses bilateral symmetry given the reduction in the stamen number (e.g., *R. pilosa* and *R. brittonii*). In *R. consanguinea*, the first whorl possesses bilateral symmetry due to the abortion of one perianth part (fig. 5).

Relative Rates of Androecium/Gynoecium Elongation

Four different relative rates of androecium/gynoecium development were recognized based on variations of the timing of stamen elongation in comparison with the gynoecium enlargement (table 1). In most flowers, the gynoecium elongates faster than the androecium from early developmental stages (e.g., *R. corymbosa*; fig. 3*C*–3*I*; type 1). In the flower of *R. scutellata*, elongation of the gynoecium and the androecium occur at a similar rate; only later does elongation of the gynoecium surpass that of the androecium (fig. 6*A*–6*D*; type 2). In *R. brittonii*, the androecium initially surpasses the gynoecium, and then the gynoecium accelerates its growth to surpass the stamens (fig. 6*E*–6*I*; type 3). Finally, in some species, something similar occurs to what is observed in *R. brittonii*, but in these cases the difference between the stamen elongation and the gynoecium elongation is greater; this situation is also inverted after the stamens are fully elongated, when the gynoecium elongates to surpass the androecium (*R. tenuis* and *R. setigera*; fig. 6*J*–6*M*; type 4).

Phylogenetic Analysis and Character Evolution

The phylogeny presented here agrees with the topology published by Thomas et al. (2009; fig. 7). The genus *Rhynchospora* is monophyletic (PP = 1 and BS = 100; note that we did not include any species of *Pleurostachys* in the analysis), with two main clades that diverged earlier, clade B and E, highly (PP = 1 and BS = 100) and weakly (BS = 58) supported, respectively. Clade B includes only species of subgenus *Haplostyleae* and is further divided into two subclades containing species *R. corymbosa* and *R. scutellata* in clade C (PP = 1 and BS = 99) and *R. terminalis* and *R. consanguinea* in clade D (PP = 0.96 and BS = 87). Clade E includes some species of subgenus *Haplostyleae* and all species of the subgenus *Rhynchospora*; this clade is further divided into subclades F and H. Clade F includes *R. brittonii*, *R. setigera*, and *R. tenuis* (PP = 0.95 and BS = 78), which are representatives of subgenus *Rhynchospora*. Clade H (PP = 1 and BS = 100) consists of two members of subgenus *Haplostyleae* (*R. riedeliana* and *R. pilosa*). The majority rule consensus tree of the BI produced an identical topology. In general, the topology is supported with BS > 78% and PP > 0.96 values, excepting clade E, which is always weakly supported no matter what method is employed.

In general, the results of the ancestral state reconstruction studies, using parsimony, maximum likelihood, and Bayesian methods, were similar; however, we found a few differences in the timing of some of the evolutionary events and the resolution of the reconstruction, depending on the method used. Ancestral character-state reconstructions are summarized below.

Number of basal sterile glumes. Character 1 (fig. 8*A*; app. C)—The presence of two basal sterile glumes (state 0) is the most likely state at the base of *Rhynchospora*. Spikelets with three basal sterile glumes (state 1) may appear twice: before the divergence of *R. terminalis*, *R. consanguinea*, *R. scutellata*,

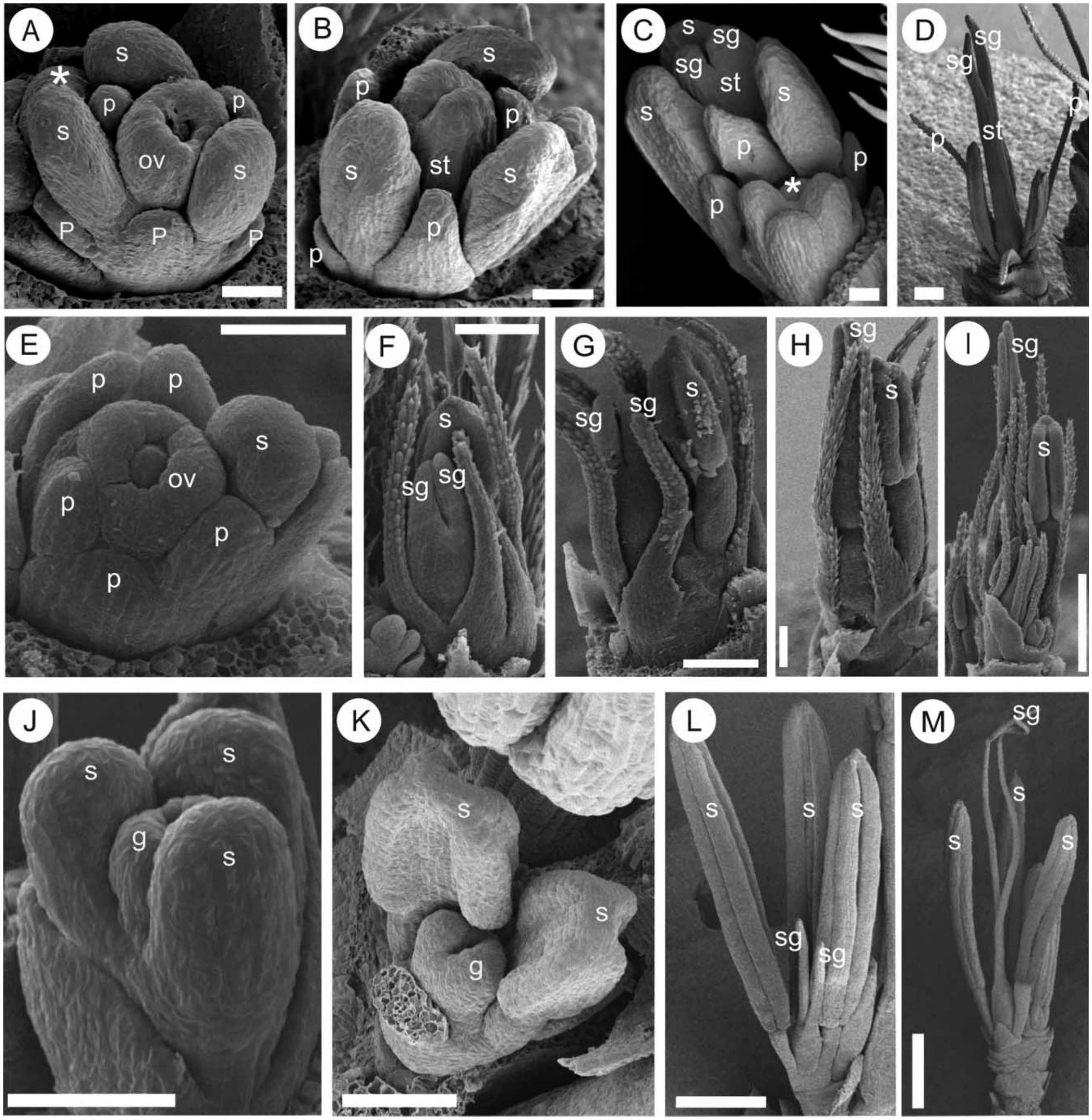

Fig. 6 Relative rates of androecium/gynoecium elongation of *Rhynchospora* flowers. Types 2, 3, and 4 are documented here, whereas type 1 is illustrated in fig. 3*D*–3*J*. Basal sterile glumes were removed. *A*, Early stage of flower development of *Rhynchospora scutellata*. *B*, *C*, Middle developmental stages of the *R. scutellata* flower, in which the androecium and the gynoecium are similar in length. *D*, Late developmental stage of *R. scutellata*, where the gynoecium surpasses the length of the androecium. *E*, Early stage of flower development of *R. brittonii*, where stamens surpass the gynoecium. *F*, *G*, Advanced stages of flower development of *R. brittonii*, where stamens continue to surpass the gynoecium length. *H*, *I*, In the end, the gynoecium of *R. brittonii* surpasses the androecium. *J*, Early stage of flower development of *R. setigera*, where the stamens are longer than the gynoecium. *K*, At middle stages of *R. setigera* flower development, the androecium continues to surpass the gynoecium in length. *L*, When stamens of the *R. setigera* flower are fully elongated, the gynoecium remains shorter than the androecium. *M*, Finally, at mature stages, the gynoecium of the *R. setigera* flower elongates to surpass the androecium. g = gynoecium, ov = ovary wall, pi = inner perianth part, po = outer perianth part, s = stamen, sg = stigma, st = style, asterisk = rachilla apex. Scale bars: *A*, *B* = 30 μm; *C* = 20 μm; *D*, *F*–*H* = 100 μm; *E*, *J*, *K* = 50 μm; *I*, *M* = 500 μm; *L* = 300 μm.

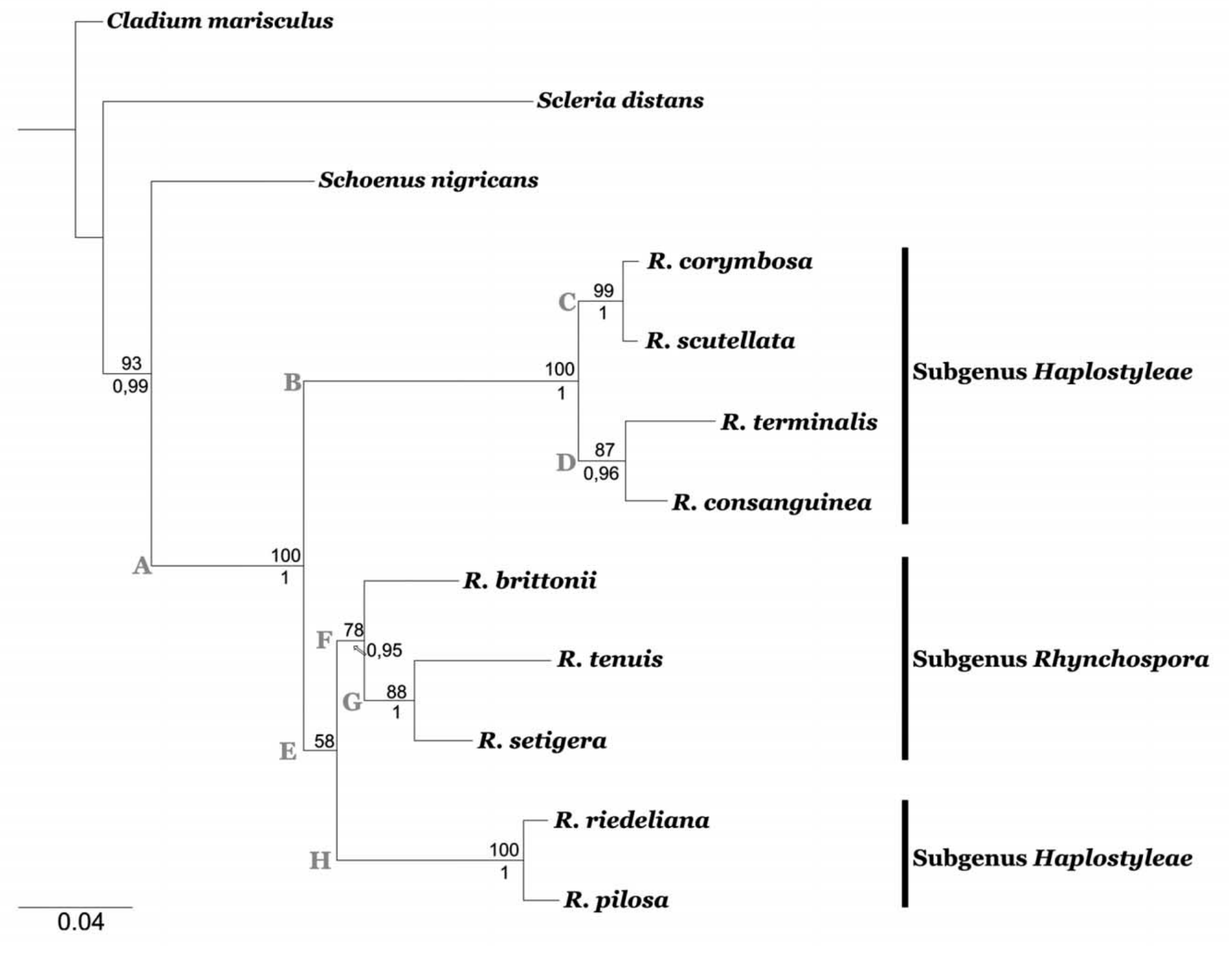

Fig. 7 Maximum likelihood tree of *Rhynchospora* obtained with PhyML 3.0. Bayesian posterior probabilities (>95) and likelihood bootstrap support values (>50) are shown below and above branches, respectively. Letters indicate clade names as assigned in this work.

and *R. corymbosa* (node B) and sometime during the diversification of subgenus *Rhynchospora* (node F or node G). Similar results were obtained under parsimony reconstructions; however, parsimony reconstructions predict that the origin of three basal glumes took place at the base of *R. tenuis* and *R. setigera* and before the diversification of *R. scutellata* and *R. corymbosa* (nodes G and C, respectively).

Spikelet type based on sexual systems. Character 2 (fig. 8*B*; app. C)—Bayesian and maximum likelihood reconstructions show that bisexual spikelets (state 1) represent the most likely state at the base of *Rhynchospora*. Bisexual spikelets were exclusively kept by subgenus *Rhynchospora* (node E and clade F). Andromonoecious spikelets (state 0) may have evolved two independent times: before the divergence of *R. terminalis*, *R. consanguinea*, *R. scutellata*, and *R. corymbosa* (node B) and at the base of the *R. riedeliana* and *R. pilosa* clade (node H). Parsimony reconstruction shows an opposite history, in which the andromonoecious spikelet may be the ancestral state for *Rhynchospora*, whereas the bisexual spikelet evolved once at the base of subgenus *Rhynchospora*. This character showed no homoplasy under parsimony reconstruction. The unisexual spikelet appears at least once in *R. terminalis* from an andromonoecious spikelet.

Number of stamens in the bisexual flower. Character 3 (fig. 8*C*; app. C)—Three stamens (state 0) is the ancestral state reconstructed using a Bayes approach. Bisexual flowers with two stamens evolved at least twice: before the divergence of *R. riedeliana* and *R. pilosa* (node H) and in *R. consanguinea*. The bisexual flower with one stamen has arisen at least once in *R. brittonii* from flowers with three stamens. Under the parsimony method, the reconstruction of the nodes of interest is uncertain. The maximum likelihood method cannot be used for the reconstruction of this character due to the polymorphic nature of *R. brittonii*.

Presence of flowers with perianth along the spikelet. Character 4 (fig. 8*D*; app. C)—Spikelets that have flowers without perianth (state 1) represent the most likely state at the base of *Rhynchospora* (node A), as well as at node E. Inside *Rhynchospora*, the perianth appears twice but in different spikelet types. Spikelets with perianth in the proximal flower and distal flowers without perianth (state 2) appear at node B, whereas spikelets that have all flowers with perianth are exclusive of

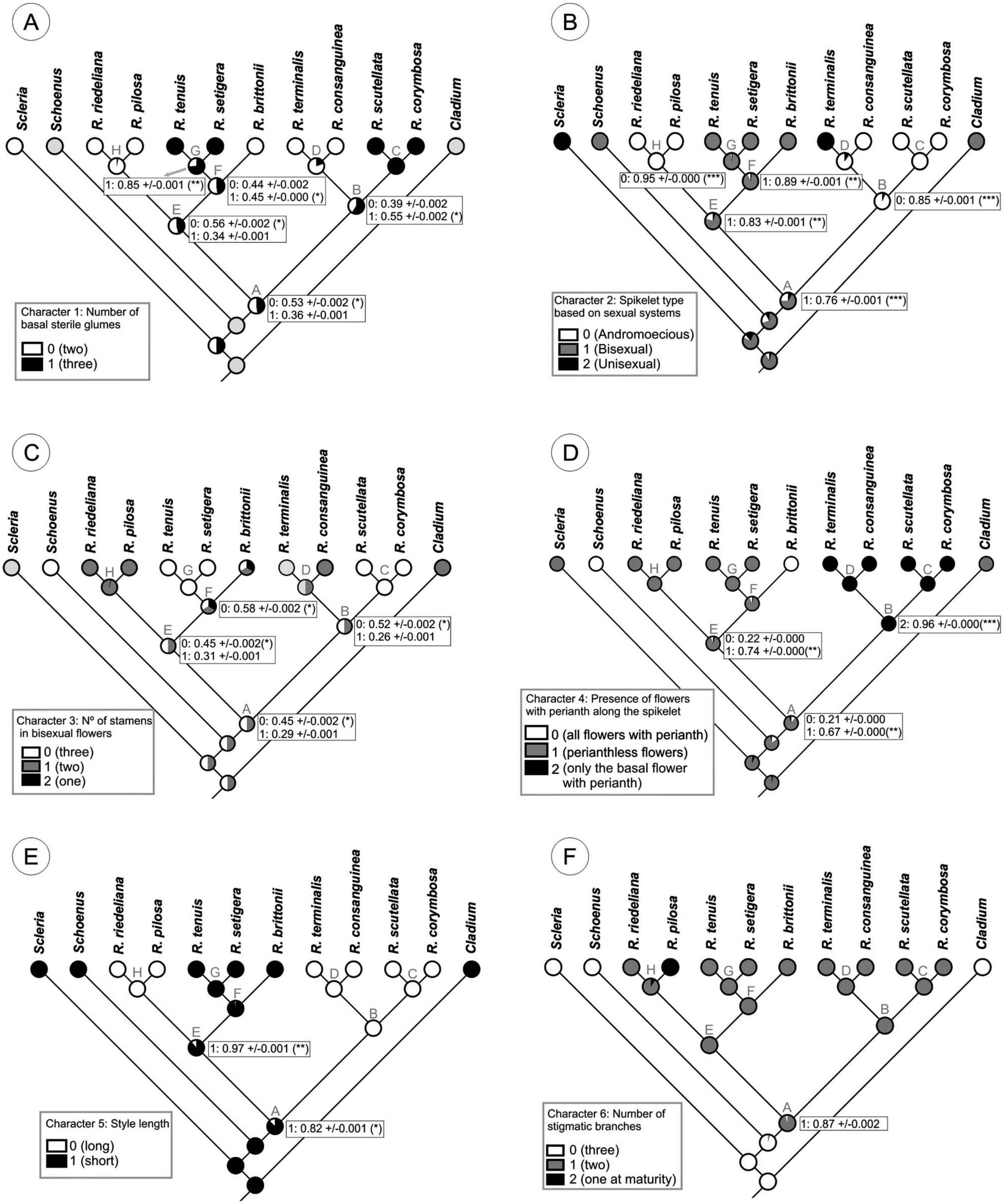

Fig. 8 Ancestral character-state reconstruction of *Rhynchospora* spikelet and flower morphology and development. Character names and their states are indicated in shaded boxes. Species mapped with gray circles represent missing data. We used the maximum likelihood tree of

R. brittonii (state 0). Parsimony reconstruction at the base of *Rhynchospora* is uncertain; for the rest of the nodes, the reconstruction is identical to the Bayesian and maximum likelihood analyses.

Style length. Character 5 (fig. 8*E*; app. C)—Bayesian and maximum likelihood methods indicate that *Rhynchospora* ancestors have flowers with a short style. Long styles appear twice at nodes B and H. Parsimony reconstruction is uncertain for nodes A and E, so the direction of the evolution of the style length cannot be assessed.

Number of stigmatic branches. Character 6 (fig. 8*F*; app. C)—The three different methods employed confirm a reduction in the number of stigmatic branches. Three stigmatic branches (state 0) is the ancestral state of the subfamily Cyperoideae. The number of stigmatic branches has been reduced to two (state 1) at the base of *Rhynchospora* and later to one (state 2) in *R. pilosa*.

Relative rates of androecium/gynoecium elongation. Character 7 (fig. 8*G*; app. C)—At nodes A, E, and F, flowers with an androecium that surpasses the gynoecium sometime during flower development (state 1) represent the most likely state, according to the Bayesian and maximum likelihood approaches. The three reconstruction methods agree that the most likely state at nodes B and H is a flower in which the androecium never surpasses the gynoecium (state 0). Parsimony reconstructions are ambiguous at the base of *Rhynchospora*; on the other hand, this method indicates that an androecium that surpasses the gynoecium sometime during flower development (state 1) is a synapomorphy of node F.

Discussion

In *Rhynchospora*, the spikelet consists of an indeterminate rachilla bearing lateral flowers in the axil of glumes. Recently, Vrijdaghs et al. (2010) addressed the structure of the spikelet based on an ontogenetic approach in subfamily Cyperoideae (Cyperaceae) and concluded that the spikelet is indeterminate (monopodial); our observations on *Rhynchospora* agree with this model of spikelet development. Furthermore, our results contrast with the conclusion of Browning and Gordon-Gray (1995); these authors, studying mature spikelets of *Rhynchospora gracillima* ssp. *subquadrata*, suggest that flowers are not axillary to glumes.

The spikelet of subfamily Cyperoideae has been described as having sterile glumes distichously or spirally arranged depending on the genus (Eiten 1976; Goetghebeur 1998; Vrijdaghs et al. 2010). The spikelet of *Rhynchospora* may have two or three basal sterile glumes and several distal fertile glumes. In systematic treatments of *Rhynchospora*, the glume arrangement in the spikelet has been considered spiral (Thomas 1992; Guaglianone 2001; Strong 2006); however, we found variations in the glume phyllotaxis within the spikelet. Interestingly, in *Rhynchospora*, basal sterile glumes develop distichously, whereas fertile glumes switch to a spirodistichous disposition. A similar phenomenon of glume phyllotaxis shift along the same spikelet rachis was found in *Scleria* (Ahumada and Vegetti 2009) and *Abildgaardia ovata* (Reutemann et al. 2009). An inverse case was observed in *Scirpus falsus* (Vrijdaghs et al. 2010; now named *Dracoscirpoides falsa* [Muasya

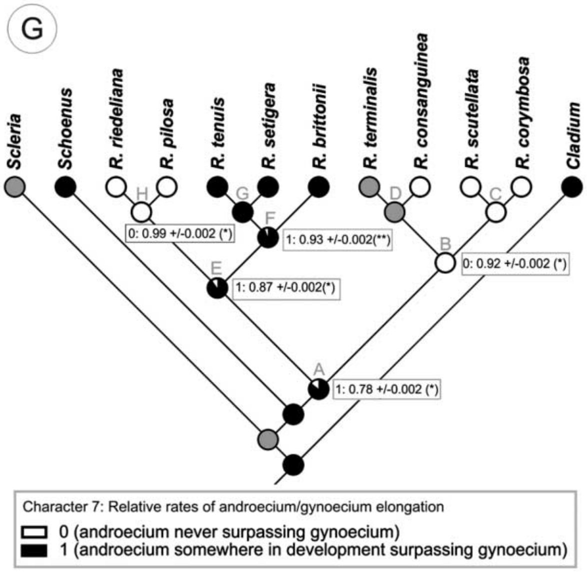

Fig. 8 (continued) fig. 7 for character optimization. Pie charts indicate the proportional likelihood obtained under the Markov one-rate model using Mesquite, version 2.01. Character 3 was reconstructed under only the parsimony criterion using Mesquite, version 2.01, due to the presence of polymorphic states. For the rest of the characters, the corresponding parsimony reconstruction is presented in appendix C, available online. Posterior probabilities (values over 20%) obtained with BayesTraits are shown at nodes of interest. The Bayes factor was performed to test the support of one particular state over the other state(s): three asterisks (BF values >5) indicate strong support; two asterisks (BF values >2) indicate positive support; one asterisk (BF values 2 > 1) indicates low support. Letters indicate node names as in fig. 7.

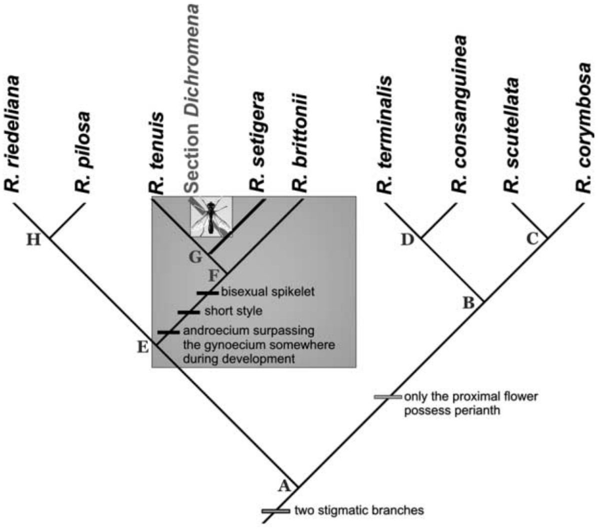

Fig. 9 Summary of *Rhynchospora* spikelet and flower character evolution. Characters that became synapomorphic under any of the reconstruction methods employed are indicated. Gray lines indicate those states inferred by Bayesian analysis, maximum likelihood, and parsimony, and black lines indicate states inferred by parsimony only. Section *Dichromena* is included in the tree (in blue) based on the topology proposed by Thomas et al. (2009); this section has only insect-pollinated species (Thomas 1984), which is illustrated by a syrphid over the blue branch. The shaded square indicates the hypothetical origin of insect-pollinated species at the base of subgenus *Rhynchospora*. Letters indicate node names as in figure 7.

et al. 2012]), where basal sterile glumes develop spirally, whereas fertile glumes switch to a distichous disposition.

Flowers of subfamily Cyperoideae develop following the scirpoid model (Vrijdaghs et al. 2005*a*, 2009). Typically, in the scirpoid model, a perfect flower begins as a primordium in the axil of a glume; two lateral stamens are the first organs initiated, followed by a third abaxial stamen. Then, three outer (opposite the stamens) and three inner (alternate with stamens) tepals appear simultaneously with the gynoecium. This tetracyclic scheme with trimerous whorls has a radial symmetry. Vrijdaghs et al. (2005*a*) found variations to this general model of flower development in *Eriophorum* and *Dulichium* due to differences in the tetracyclic scheme. In general, *Rhynchospora* flowers follow the scirpoid pattern proposed for the subfamily Cyperoideae, except for variations in the symmetry and timing of floral organ development. *Rhynchospora* flowers with two stigmatic branches or a reduced number of stamens have a bilateral symmetry instead of a radial one, as the model predicts; only *Rhynchospora* male flowers show a radial symmetry. Interestingly, in *Rhynchospora*, the outer whorl of perianth initiates before the gynoecium, whereas in the scirpoid model, the gynoecium appears simultaneously with the two whorls of perianth.

Evolution of Spikelet and Flower Morphology and Development in Rhynchospora

The phylogeny herein produced (fig. 7) is consistent with the phylogeny proposed by Thomas et al. (2009). When the morphological and developmental characters were analyzed on the *Rhynchospora* tree, we found that spikelet and floral traits indeed are consistent with the evolution of several clades. In addition, some of the spikelet and floral character modifications may be correlated with the evolution of *Rhynchospora* mating and pollination systems and fruit dispersal mode, as explained below (fig. 9). q9

We observed that the variation in the number of basal sterile glumes is correlated with the molecular phylogeny of *Rhynchospora*. The ancestor of the genus may have had a spikelet with two sterile glumes. Later, the number of sterile glumes has increased to three at least two independent times, supporting the origin or diversification of subgenus *Rhynchospora* and the lineage consisting of *R. terminalis*, *R. consanguinea*, *R. scutellata*, and *R. corymbosa*.

One of the main concerns of botanists since Darwin's time has been the changes in the sexuality of flowers and sexual systems of angiosperms. Although it is not clear whether the bisexual flower is the angiosperm ancestral state (Endress and Doyle 2009), it is known that unisexual flowers have originated multiple times, from bisexual flowers, in almost 10% of angiosperms (Barret 2002; Mitchell and Diggle 2005; Diggle et al. 2011). In this work, two different histories on the evolution of flower sexuality were recovered, depending on the methodology. Indeed, Bayesian analyses and maximum likelihood reconstructions estimated that the ancestral state of *Rhynchospora* is a bisexual spikelet (having only bisexual flowers). By contrast, the parsimony method suggested that the ancestral state of the genus may be an andromonoecious spikelet (consisting of a proximal bisexual flower and several distal male flowers). Several spikelet types, based on the sex of the flowers, have been described for Cyperoideae. Two distinct spikelet types had previously been characterized for *Rhynchospora*, the andromonoecious and the bisexual (Kükenthal 1949, 1950, 1951, 1952; Koyama 1972; Thomas 1992; Guaglianone 2001; Strong 2006). We have found an additional *Rhynchospora* spikelet type, named here a unisexual spikelet, that has unisexual flowers exclusively. The ancestral state reconstruction analyses may indicate that the unisexual spikelet evolved only later in the history of the genus from an andromonoecious spikelet; this conclusion needs further corroboration with a broader sampling. While in some of the Cyperoideae genera, one spikelet type predominates (e.g., *Cyperus* or *Eleocharis*; Eiten 1976), others such as *Rhynchospora*, *Carex* (Goetghebeur 1998), and *Scleria* (Ahumada and Vegetti 2009) have diversified their spikelet structure, changing the sex of the flowers. Given the uncertainties of the ancestral state reconstruction at the base of the genus, we were not able to infer the direction of the evolution of such changes in *Rhynchospora*; however, considering the importance of sexual systems for reproduction, we presume that the evolution of the spikelet types of *Rhynchospora* may denote an evolution of the *Rhynchospora* pollination mode, mating system, and even fruit dispersal mode.

The loss of stamens seems to be correlated with the sex of the flower of *Rhynchospora*. Bisexual flowers may develop one, two, or three stamens, and male flowers always have three stamens. The reconstruction of this floral trait was equivocal at several nodes. Usually in Cyperoideae, the stamen number does not vary among flowers of different sexes. Few exceptions to this general trend have been reported. For instance, the male flower of *Pycreus pumilus* (Vrijdaghs et al. 2009) has more stamens than bisexual flowers. The reduction of stamens in the bisexual flower might possibly also reduce the chance of autogamy and promote outcrossing.

Some of the genera of Cyperaceae are characterized by species that bear flowers with a perianth, whereas other genera or entire tribes have no perianth. It has been postulated that in sedges, the perianth may play an important role in fruit dispersion (Bruhl 1995; Goetghebeur 1998). Only a few genera of sedges, such as *Rhynchospora*, include species both with and without perianth; other examples were registered in *Eleocharis*, *Fuirena*, and *Schoenus* (Goetghebeur 1998). According to our results, flowers with a perianth evolved at least twice, while the ancestor of *Rhynchospora* may have flowers lacking a perianth. This character supports the divergence of the two main lineages of *Rhynchospora*; the presence of perianth at the proximal flower of the spikelet is a synapomorphy of clade B, whereas flowers without perianth belong to clade E. The abortion of only one tepal from the outer whorl in *R. consanguinea* is another interesting and new finding for Cyperaceae; given that we examined only one specimen, we cannot be certain that this is a stable condition, and further observations in this and other species of the genus and family are needed to confirm the occurrence of organ abortions in Cyperaceae.

In our study, we interpreted the style as short or long and undivided. Here the short style is a synapomorphy of subgenus *Rhynchospora*, and it seems to be correlated with the sexual system. While andromonoecious and unisexual spikelets have flowers with a long style, bisexual spikelets (exclusive of subgenus *Rhynchospora*) develop flowers with a short style. Whether this trait has implications for the mating system in *Rhynchospora* is an open question.

The trimerous condition of floral organs in Cyperoideae has faced reductional trends in several genera (Vrijdaghs et al. 2004, 2005*a*, 2005*b*, 2006, 2009). In *Rhynchospora*, the number of stigmatic branches was reduced to two branches from the Cyperoideae ancestor, which had three stigmatic branches. In this work, we have reported an additional and extreme case of stigmatic branch reduction in *R. pilosa*, in which only one stigmatic branch reaches maturity, while the other one aborts. Similarly, Vrijdaghs et al. (2011) have reported that *Pycreus flavescens* (which is placed in the tribe Cypereae) has two stigmatic branches growing at different rates (one is retarded with respect to the other), but finally both branches complete their development.

Our finding of different relative rates of androecium/gynoecium elongation in *Rhynchospora* stresses the importance of a comparative approach in close relatives, also in Cyperaceae. According to the parsimony reconstruction, an androecium that surpasses the gynoecium sometime during flower development is a synapomorphy of subgenus *Rhynchospora*. Flowers in which the gynoecium is never surpassed by the androecium during development appear two independent times. The flower may be protogynous when the androecium is always shorter than the gynoecium during development, whereas it may be protandrous when the androecium surpasses the gynoecium at some stages of development. Interestingly, protogynous species are associated with wind pollination, whereas protandrous species are commonly animal pollinated (Sargent and Otto 2004; Friedman and Barrett 2008, 2009). Given this, the recognition of this trait may be an important first step toward understanding the pollination system in *Rhynchospora*.

Systematic Implications

Although the previous subgeneric classification based on the morphology of the style is not consistent with the phylogeny of Thomas et al. (2009) and the one presented here, our work showed that some morphological and developmental characters are indeed correlated with the molecular phylogeny of *Rhynchospora* (fig. 9). The presence of a perianth in the proximal flower of andromonoecious spikelets at node B and the absence of perianth in all flowers of the spikelets at node E support the divergence of these clades. The short style, the spikelet with only bisexual flowers, and the flowers where the androecium surpasses the gynoecium sometime during development are synapomorphies for the clade that includes *R. brittonii*, *R. setigera*, and *R. tenuis* (node F). We presume that these characters would be of help in a future revision of the systematics of *Rhynchospora*.

Spikelets and Floral Traits That Are Related with an Entomophilous Pollination in Rhynchospora

The pollination system of *Rhynchospora* has changed from anemophilous to entomophilous at least once in the derived clade that includes all *Dichromena* species (Thomas et al. 2009). We found similarities among insect-pollinated *Dichromena* species and other members of subgenus *Rhynchospora* studied in this work. Indeed, *Dichromena* species have bisexual spikelets (producing three or more achenes per spikelet) and have flowers with short styles, such as *R. brittonii*, *R. setigera*, and *R. tenuis* (clade F). Moreover, *R. setigera* is sister to *Dichromena* (Thomas et al. 2009), both having capitate inflorescences, white glumes, and flowers without perianth (fig. 9). The floral similarities between *Dichromena* species and *R. brittonii*, *R. setigera*, and *R. tenuis* indicate that at least part of the subgenus *Rhynchospora* may have diversified from the rest of *Rhynchospora* through changes in its spikelet and flower morphology and specialization of its pollination mode. Several floral and developmental traits studied here, such as the presence of a short style, the relative rate of androecium/gynoecium elongation, bisexual spikelets, and flowers without perianth, may support the origin of subgenus *Rhynchospora* (fig. 9); interestingly, the bisexuality of flowers and the protandrous condition of flowers are related to entomophilous pollination (Sargent and Otto 2004; Friedman and Barrett 2008, 2009; Givnish et al. 2010; Soza et al. 2012). In particular, Wragg and Johnson (2011) suggested that it is not unexpected that insect-pollinated Cyperaceae evolve from wind-pollinated plants that have both sexes in close proximity in space and time; other insect-pollinated Cyperoideae species were reported to have bisexual spikelets (Thomas 1984; Wragg and Johnson 2011); however, it must be remarked that so far the main focus was put on the subfamily Cyperoideae and that subfamily Mapanioideae should receive more attention in the future. In this sense, the presence of bisexual spikelets (in which both sexes are in close proximity) in subgenus *Rhynchospora* may also represent a step toward an entomophilous pollination. Consequently, sexual systems in the Cyperaceae spikelet could be considered another relevant trait correlated with the change to entomophilous pollination in the family. According to our observations, the presence of protandrous flowers may occur in some species of subgenus *Rhynchospora* where the androecium develops faster than the gynoecium. The implication of flowers without perianth and the short style in insect-pollinated species of Cyperaceae has not been investigated so far (Thomas 1984; Friedman and Barrett 2008; Wragg and Johnson 2011).

Concluding Remarks

In this work, we provide sequences of floral organogenesis of nine species of *Rhynchospora* that differ in the presence/absence of perianth, number of stamens in bisexual flowers, number of stigmatic branches, style length, and sexuality of spikelets. We found four different relative rates of androecium and gynoecium elongation, which may indicate the presence of protandrous and protogynous species within the genus. The phylogenetic analysis carried out here indicates that some morphological and ontogenetic features of flowers and spikelets support the diversification of several lineages. The bisexual spikelet and flowers in which the androecium develops faster than the gynoecium may represent important steps for the transition from a wind- to insect-pollination system in the genus.

Acknowledgments

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Appendix A

Voucher information of studied taxa. Specimens are deposited at the "Ingeniero A. Ragonese" herbarium (SF). Taxon: voucher specimen (collection country), accession number. ***R. brittonii* Gale**: LL 63 (Argentina), KC11177. ***R. consanguinea* (Kunth) Boeck.**: SM 341 (Brasil), KC111770. ***R. corymbosa* (L.) Britton**: AGR 140 (Argentina), KC111768; LL 14 (Argentina). ***R. pilosa* Boeck.**: SM 342 (Brasil), KC111775. ***R. riedeliana* C. B. Clarke**: SM 344 (Brasil), KC111776. ***R. setigera* (Kunth) Boeck.**: AGR 151 (Argentina), KC111773; AGR 154 (Argentina); AGR 165 (Argentina). ***R. scutellata* Griseb**: LL 03 (Argentina), KC111769; AGR 58 (Argentina). ***R. tenuis* Link**: AGR 131 (Argentina), KC111774; LL 20 (Argentina). ***R. terminalis* (Nees) Steud**: SM 343 (Brasil), KC111771.

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QUERIES TO THE AUTHOR

q1. I edited your article for clarity and agreement with journal style, and minor revisions were made without comment. Please review the text and advise whether any of my changes are unacceptable. Any layout problems or errors you see will be corrected after the proof is returned.

q2. Your appendix 1 was split into print appendix A, containing the voucher information, and online appendix B, containing all of the figures. I have updated each original reference to appendix 1 to include both appendix A and appendix B, but please update as necessary if you do not need to cite both appendixes in each case. Appendix C now contains the character and state information and the accompanying figure.

q3. In the Material and Methods section, please be sure the name of the manufacturer and the manufacturer's location is provided for all of the equipment used.

q4. Figure 7 and table C1 both mention "Cladium marisculus," which appears to be a misspelling. Okay to correct those to match the spelling here, "Cladium mariscus"?

q5. Should this be "Bayesian inference (BI)"? If not, what does BI stand for?

q6. Is citation of appendixes A and B correct here, or should it also include appendix C?

q7. In the table 1 note, figure 8, and figure C1*B*, should "andromoecy/andomoecious" read "andromonoecy/andromonoecious"?

q8. The figure 3 legend references "arrows" in panel D, although panel D does not include any. Please review and advise re. the appropriate edit.

q9. The figure 9 legend references a "blue branch," but the image is grayscale. How would you like revise the legend? Also, in the graphic, which should it be, "only the proximal flower possesses perianth" or "only the proximal flowers possess perianth"?