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Research Article

# Nuclear phylogeny and hypothesized allopolyploidization events in the Subtribe Otachyriinae (Paspaleae, Poaceae)

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Otachyriinae Butzin, one of the three subtribes within the Paspaleae tribe of grasses, includes seven genera and approximately 35 species. Interestingly, this subtribe comprises species with $C_3$, $C_3$-$C_4$ intermediate, and $C_4$ photosynthesis. The circumscription of Otachyriinae has changed through time, and still varies among treatments in current use. The monophyly of this subtribe has been recovered in previous studies based on plastid markers. A phylogenetic study taking into account polyploidy and a low-copy nuclear gene (LCNG) is still lacking in Paspaleae. A phylogeny including data from a LCNG is paramount to uncover reticulated evolution within the group. The purpose of the present study was to reconstruct the evolutionary history of Otachyriinae, based on both chloroplast and LCNG DNA with a focus on polyploidization. Several incongruences between gene trees allowed us to explore relationships between diploid and polyploid taxa. Our study identified several promising topics for future studies: genetic allopolyploidy and autopolyploidy was here documented using the characteristic pattern of double-labelled gene trees. The molecular evidence indicates that at least 40% of species of Otachyriinae show phylogenetic signature of polyploidy (16 taxa appear double-labelled in the nuclear gene trees); furthermore, the results support an allopolyploid origin of at least nine taxa in the subtribe: *Rugoloa polygonata*, a species with unknown photosynthetic pathway, the proto-kranz species *Steinchisma laxum* and *Rugoloa hylaeica*, and the $C_3$ species *Hymenachne felliana*, *H. grumosa*, *H. hemitomon*, *H. donacifolia*, *H. pernambucense*, and *P. grande*. Also, our results confirm that the $C_4$ genus *Anthaenantia* is unambiguously monophyletic and show that *Anthaenantia lanata* is an autopolyploid. We recognized six genera within subtribe Otachyriinae: *Anthaenantia*, *Hymenachne*, *Otachyrium*, *Plagiantha*, *Rugoloa*, and *Steinchisma*. Finally, *Panicum* species with historically ambiguous placements, i.e. *P. condensatum*, *P. grande*, *P. harleyi*, *P. leptachne*, *P. longum*, and *P. stagnatile* were transferred to the genus *Hymenachne*.

**Key words:** apo1, grasses, ndhF, polyploid, photosynthesis evolution, taxonomy

## Introduction

Otachyriinae Butzin, one of the three subtribes within the Paspaleae tribe, consists of seven genera with approximately 35 species. Species of this subtribe are widely distributed in wet habitats in the tropics of America, Asia, and Australia. Subtribe Otachyriinae is morphologically characterized by having spikelets usually arranged in unilateral branches, with the lower glume shorter than the upper glume and lower lemma, and upper anthecium membranous to indurate; it includes species of *Anthaenantia* P. Beauv., *Hymenachne* P. Beauv., '*Panicum incertae sedis*' species of sect. *Laxa*, *Plagiantha* Renvoize, *Otachyrium* Nees, *Rugoloa* Zuloaga, and *Steinchisma* Raf.

Species of Otachyriinae include $C_3$, $C_3$-$C_4$ intermediate, and $C_4$ species. In the 1980s, the anatomy, biochemistry, and physiology of some *Panicum* species, now considered in the Otachyriinae were extensively characterized (Bouton, Brown, Bolton, & Campagnoli, 1981; Brown, Bouton, Evans, Malter, & Rigsby, 1985; Brown, Bouton, Rigsby, & Rigler, 1983; Edwards, Ku, & Hatch, 1982; Holaday & Chollet, 1983; Hylton, Rawsthorne, Smith, Jones, & Woolhouse, 1988; Morgan, Brown, & Reger, 1980; Renvoize, 1987; Sternberg, Deniro, Sloan, & Black, 1986). Interestingly, Monson, Rawsthorne, and co-workers (Monson &

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Moore, 1989; Rawsthorne & Bauwe, 1998) proposed the model of the gradual progression from $C_3$ towards $C_4$ photosynthesis based on the leaf anatomical variation observed in this group. Also, new insights in $C_4$ evolution have highlighted the importance of the species included in this subtribe to serve as a model for $C_4$ grass evolution (Khoshravesh et al., 2016; Sage, Khoshravesh, & Sage, 2014). The Otachyriinae was strongly supported as monophyletic both when analysing the *ndhF* dataset independently (Acosta, Scataglini, Reinheimer, & Zuloaga, 2014; Aliscioni, Giussani, Zuloaga, & Kellogg, 2003) or combined with morphology (Morrone et al., 2012). Although these studies contributed significantly to the knowledge of evolutionary relationships within Otachyriinae, the phylogeny of this subtribe is still not completely resolved. Some questions remain unanswered while others have arisen as a result of newly published data that uncovered novel and/or complex patterns of relationships.

The circumscription of Otachyriinae has changed through time, and still varies among treatments in current use (Acosta et al., 2014; Grande Allende, 2014; Kellogg, 2015). Several species included in Otachyriinae previously belonged to *Panicum* sect *Laxa* Hitchcock & Chase ex Pilger (Pilger, 1931). Zuloaga, Ellis, and Morrone (1992) included 13 species in the section. Later, Aliscioni et al. (2003) and Morrone et al. (2012) found that sect. *Laxa* is polyphyletic, species of this being morphologically similar to taxa of the Otachyriinae, such as *Steinchisma* and *Hymenachne.* Therefore, some *Panicum* taxa have been transferred to genera of the Otachyriinae, such as *Hymenachne*, and *Steinchisma* (Acosta et al., 2014; Aliscioni et al., 2003); also, the new genus *Rugoloa*, including '*incertae sedis*' species of *Panicum* was established within the subtribe (Acosta et al., 2014). Nevertheless, there remain *Panicum* species of sect. *Laxa* in need of a clear systematic position (i.e., *Panicum bresolini* L.B. Sm. & Wassh., *P. condensatum* Bertol., *P. longum* Hitchc. & Chase, and the ungrouped *P. grande* Hitchc. & Chase), whose taxonomic placement is unresolved because of the lack of plant material or herbarium specimen observation. Additionally, some taxonomic discrepancies still need to be analysed within the subtribe. Kellogg (2015) considered the genera *Plagiantha* and *Steinchisma* within the genus *Otachyrium*, based on phylogenetic results and that all three genera share a distinctive hardened palea. Also, Grande Allende (2014) treated some species within the Otachyriinae and proposed several new combinations at the species level, the new genus *Aconisia* and new infrageneric synonyms previously included in *Panicum*. However, Grande Allende's work lacked observations of plant material and/or herbarium specimens, while this author did not include any appropriate analytical method (or methods) to test his hypothesis and conclusion.

A reconstructed phylogeny helps guide interpretation of the evolution of organismal characteristics, providing hypotheses about the lineages in which traits arose and under what circumstance, thus playing a vital role in studies of adaptation and evolution. Although the monophyly of Otachyriinae has been recovered in previous studies (Acosta et al., 2014), some of the currently recognized genera resulted para- and polyphyletic, such as *Otachyrium* and *Hymenachne* respectively (Acosta et al., 2014).

Published chromosome counts are available for several Otachyriinae species (Bouton et al., 1981; Davidse & Pohl, 1972, 1974, 1978; Hidalgo, Caponio, & Norrmann, 2007; Morrone, Hunziker, Zuloaga, & Escobar, 1995; Pohl & Davidse, 1971; Zuloaga, Morrone, Vega, & Giussani, 1998). Based on these data, Otachyriinae shares, with the rest of the Paspaleae tribe, a base chromosome number of $x = 10$ (Morrone et al., 2012). Furthermore, these cytological studies showed that some species included in the Otachyriinae were identified as diploids while others were polyploids. Polyploidy, the possession of more than two complete genomes, is a major force in plant evolution known to affect the genetic and genomic constitution and the phenotype of an organism; as a result, polyploidy has a strong influence in the ecology and geography as well as in lineage diversification of taxa (Weiss-Schneeweiss, Emadzade, Jang, & Schneeweiss, 2013).

Phylogeny reconstruction in clades including polyploid species groups is often difficult due to the reticulate nature, considering that allopolyploids combine effects of genome doubling (polyploidy) together with genome merger (hybridization) (Estep et al., 2014; Weiss-Schneeweiss et al., 2013). The parental origins and evolutionary history of polyploids could be determined with the combined use of nuclear and organelle phylogenies. Although the internal transcribed spacer of the ribosomal genes (ITS) is widely used, its high copy number and concerted evolution make it inadequate for this purpose (Álvarez & Wendel, 2003).

Earlier attempts to resolve relationships within other lineages of Paspaleae using chloroplast and nuclear markers (such as ITS and ETS), produced poorly supported phylogenies (Scataglini, Zuloaga, Giussani, Denham, & Morrone, 2014) or unresolved intergeneric relationships (Mathews, Spangler, Mason-Gamer, & Kellogg, 2002). More than 80% of grass species have undergone polyploidy some time during their evolutionary history (Stebbins, 1985); therefore, a failure to account for polyploidy may be one important reason for the difficulty

of resolving phylogenetic relationships in grasses (Estep et al., 2014). Since chloroplasts are mostly uniparentally inherited, they cannot recover reticulations caused by allopolyploids; only nuclear sequence data have the power to provide this information (Kellogg, 2016).

A phylogenetic study taking into account polyploidy, based on several individuals per species, and a low-copy nuclear gene (LCNG) is still lacking in Paspaleae subtribes, which severely restricts evolutionary studies in these taxa. Due to the fact that polyploidy and reticulate evolution are common in grasses and diploids/polyploids have been registered for species included in Otachyriinae, new data from LCNG will provide important new insights about the history of the Otachyriinae.

The purpose of the present study was to reconstruct the evolutionary history of the Otachyriinae, with a focus on possible polyploidization events. We used a phylogenetic approach to obtain insights from chloroplast NADH dehydrogenase subunit F (*ndhF*) and nuclear Aberrant Panicle Organization 1 (*apo1*) markers, as a framework to detect potential events of reticulate evolution within the subtribe. We also point out incongruences between gene trees that allowed us to explore the relationships between diploid and polyploid taxa. Moreover, we provide diagnostic morphological characters for the genera recognized in our comprehensive species-level phylogeny derived from a combined analysis of *ndhF* and *apo1* markers, and propose a revised generic classification of the Otachyriinae. The molecular sampling in this study improves our understanding of the affinities within all major clades, and clarifies the taxonomic affiliations of *Panicum* species with historically ambiguous placements.

# Materials and methods

## Taxon sampling

The data matrix included 57 accessions; nine outgroup species and 48 specimen vouchers representing all genera of the Otachyriinae, i.e., *Anthaenanthia*, *Hymenachne*, *Otachyrium*, *Plagiantha*, *Rugoloa*, and *Steinchisma*, the *incertae sedis* species of *Panicum* sect. *Laxa* (*P. condensatum*, *P. harleyi* Salariato, Morrone & Zuloaga, *P. leptachne* Döll, *P. longum*, and *P. stagnatile* Hitchc. & Chase) and the ungrouped species *Panicum grande*. Vouchers are listed in Appendix S1, see online supplemental material, which is available from the article's Taylor & Francis Online page at http://dx.doi.org/xx.xxxx/xxxxxxxx.xxxx.xxxxxx).

**DNA sequencing and processing**. Total genomic DNA was extracted from silica-dried leaves using a modified CTAB protocol (Doyle & Doyle, 1987). For herbarium specimens, DNA was isolated using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's recommendations.

The chloroplast marker *ndhF* gene was amplified and sequenced in four steps using the following primers specified by Olmstead and Sweere (1994) and Aliscioni et al. (2003): 5F-536R, 536F-972R, 972F-1666R, and 1666F-3R. The PCR reactions for the chloroplast marker were performed as described in Acosta et al. (2014).

The nuclear *apo1* gene was amplified using the primers specified by Estep et al. (2012) subcloned in a pGEM-T Easy Vector and sequenced. The *apo1* primers occasionally amplify multiple loci as a single band, so a second set of primers (F2: 5'-ACC TCC CCT TCT TYG CCT - 3' and R2: 5'- GCC ACG TCG AAC ACV AGM A - 3') were designed for nested PCR using the same thermocycler conditions for greater specificity. The PCR reactions were performed in 25 µL final volumes with 50–100 ng of template DNA, 0.2 µM of each primer, 25 µM dNTP, 5 mM $MgCl_2$ 1× buffer and 0.3 units of Taq polymerase provided by Invitrogen Life Technologies. For most of the species, PCR was carried out using the following parameters: one cycle of 94 °C for 5 min, 39 cycles of 94 °C for 30 s, 55 °C for 1 min, and 72 °C for 1 min 30 s, and a final extension cycle of 72 °C for 10 min. The PCR products were gel purified using the gel purification kit of PB-L (Buenos Aires, Argentina) and ligated into Promega (Madison, Wisconsin) pGEM-T Easy vectors following the manufacturer's protocols. Ligations were transformed in DH5α competent cells. After heat-shocking the cells for 2 minutes at 42 °C, 1 ml LB medium was added to the transformation and cultures were allowed to stabilize in a shaker at 37 °C for 2 hours. The cultures were then plated on LB-agar plates (containing Sigma-Aldrich (Steinheim, Germany) Ampicillin, Promega (Madison, Wisconsin) X-gal and Promega IPTG for blue/white screening) and incubated at 37 °C overnight. Between five and 10 white colonies from each transformation were selected. Selected colonies were PCR-amplified using Pegasus taq polymerase (PB-L, Buenos Aires, Argentina) and the vector primers M13F and M13R to confirm the presence of the insert. Plasmids were isolated for positive clones using the purification plasmid kit (PB-L, Buenos Aires, Argentina). Manufacturer's protocols were followed for all steps. At least five positive clones for each PCR product were sequenced in both directions. Sequencing reactions using the T7 (forward) and M13-pUC (reverse) universal primers were performed by Macrogen using the ABI PRISM BigDye terminator cycle sequencing kits with AmpliTaq DNA polymerase (Applied Biosystems, Seoul, Korea). Chromatogram files were trimmed of vector and low

quality sequences and reverse and forward sequences for each clone were assembled and edited using the program MEGA v.6 (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013). All good quality sequences for each marker were then aligned using ClustalX v2 (Larkin et al., 2007) under the default options.

## Data matrix assembly

The previously published DNA matrix of the chloroplast *ndhF* marker (Acosta et al., 2014) was completed with sequences of three new added species (*P. longum*, *P. condensatum*, and *P. grande*) and sequences for 21 new voucher accessions (Appendix S1, see supplemental material online).

The initial dataset for the *apo1* nuclear locus included 196 sequences in total. Automated methods included in the Recombination Detection Program RDP4 v4.36 (Martin, Murrell, Golden, Khoosal, & Muhire, 2015) were used, in order to account for recombination events, to identify the presence of chimerical sequences. Although the complete *apo1* gene matrix did not include recombinant sequences, this dataset contained numerous redundant clones. To reduce the number of redundant sequences to one per paralogue per locus, a preliminary phylogenetic analysis, with all clones of all taxa, was conducted in RAxML (Stamatakis, Hoover, & Rougemont, 2008). Based on the maximum likelihood preliminary analysis, clones that formed a clade and that differed by fewer than five nucleotides were inferred to represent a single locus and were combined into a single majority-rule consensus sequence using the perl script clone_reducer (github.com/mrmckain) as described in Estep et al. (2014). Clones that did not meet these criteria were kept separate through subsequent analyses. The final dataset contained 98 sequences.

Voucher specimen and its corresponding GenBank accession numbers for *ndhF* and *apo1* sequences used in the phylogenetic analyses (described below) are listed in Appendix S1 (see supplemental material online). All aligned matrices are available online from TreeBase (Study Accession URL: http://purl.org/phylo/treebase/phylows/study/TB2:S23129).

## Phylogenetic analyses

The chloroplast *ndhF* marker and the nuclear marker *apo1* were analysed separately first using Parsimony (MP), Maximum likelihood (ML) and Bayesian inference (BI). In these analyses gaps were treated as missing data.

For MP analysis, tree searches were generated in TNT v1.1 (Goloboff, Farris, & Nixon, 2008) using heuristic searches with 1,000 random addition sequences, tree bisection and reconnection (TBR) branch swapping, and holding 10 trees per replicate. Generated trees were then submitted to a new round of TBR branch swapping to completion. The non-parametric bootstrap of Felsenstein (1985), with 1,000 replicates, was used to assess branch support.

Analyses BI and ML were performed using the CIPRES Science Gateway V.3.3 (Miller, Pfeiffer, & Schwartz, 2010). The best-fit model of nucleotide substitution for each marker was identified using the Akaike information criterion (AIC) implemented in jModeltest 2.1.3 (Darriba, Taboada, Doallo, & Posada, 2012) (*ndhF*: GTR + G, *apo1*: GTR + I + G).

The ML analyses were conducted in RAxML v8.2.4 (Stamatakis, 2014) using non-parametric bootstrap (BS) analysis and searches for the best-scoring ML tree in a single run (Stamatakis et al., 2008). We performed 1,000 rapid bootstrap inferences and, thereafter, a thorough ML search under the GTRGAMMA (*ndhF*) and the GTRGAMMAI (*apo1*) models.

Additionally we tested the monophyly of species and genera associated to their current generic classification on plastid and nuclear trees using the SH test (Shimodaira & Hasegawa, 1999) implemented in RAxML v8.2.4 (Stamatakis, 2014). Searches of constrained topologies were conducted in RAxML with 1,000 replicates and the models used above, and the significance of differences between the best ML unconstrained and constrained trees was determined using 10,000 BS replicates and rejecting the hypothesis when $P < 0.01$.

The BI was carried out with MrBayes v.3.2.1 (Ronquist et al., 2012) implementing two parallel runs of four simultaneous Markov chains for 10 million generations, sampling every 1,000 generations and using the default parameters. Convergence of the runs was assessed by checking the status of parameters in Tracer v.1.5 (Rambaut & Drummond, 2007) to ensure the stationarity of each run. Likelihoods of the trees produced by each run were analyzed graphically using Tracer v.1.5 (Rambaut & Drummond, 2007) and, after discarding the initial 2,500 trees of each run as burn-in (25%), the remaining trees were summarized in a Maximum clade credibility tree (MCCT) including the posterior probabilities (PP) as branch support estimates.

## Inference of allopolyploids

The positions of different *apo1* copies in the nuclear gene tree obtained were used for inference of parental progenitors of polyploids species. If sequences from a polyploid species grouped within different clades, this

was interpreted as an indication for allopolyploidy, while autopolyploidy was inferred if all sequences of a polyploid were in a single clade (Brassac & Blattner, 2015; Estep et al., 2014; Kellogg, 2016). For statistical support, the SH test (described above) was used to assess the monophyly of *apo1* gene copies for each allopolyploid species inferred. Also, chloroplasts are inherited maternally in most angiosperms (Reboud & Zeyl, 1994), so phylogenies obtained by cpDNA markers have been used for determining a maternal parent of a polyploid (Brassac & Blattner, 2015; Nishikawa, Salomon, Komatsuda, von Bothmer, & Kadowaki, 2002). Thus, putative male and female crosses resulting in allopolyploid taxa within Otachyriinae were inferred comparing the *ndhF* phylogeny with the phylogeny obtained from the *apo1* dataset. Chromosome counts presently known for Otachyriinae species used to argue about the ploidy level of each taxon (see Discussion section) are summarized in Table S1 (see supplemental material online).

### Phylogenetic analyses of the combined dataset

To take into account the allopolyploid taxa, for combined analyses including both datasets, a pruned *apo1* gene matrix was constructed by reducing it to a set of sequences comparable with the *ndhF* dataset. For each allopolyploid taxa identified, we selected the *apo1* sequences that were most similar in position to the *ndhF* phylogeny. This biases the results in favour of compatibility of trees but these analyses allowed us to analyse possible incongruences between gene trees excluding the allopolyploidization events. Topological discordance among gene trees due to incomplete lineage sorting, and possibly other phenomena can cause concatenation analyses to fail (Pérez-Escobar, Balbuena, & Gottschling, 2016; Salichos, Stamatakis, & Rokas, 2014). To avoid this, a coalescent-based estimation of the species tree, implemented in BEAST (Larget, Kotha, Dewey, & Ané, 2010) and Bayesian Concordance Analysis, implemented in BUCKy (Ané, Larget, Baum, Smith, & Rokas, 2007), were done. Then, after discarding the incongruent sequences due to allopolyploid taxa and assuming that all topological discordance between the cpDNA dataset and the pruned nuclear dataset is caused by incomplete lineage sorting (ILS), a coalescent-based estimation of the species tree was performed using BEAST 1.8.3 (Drummond, Suchard, Xie, & Rambaut, 2012). We applied the lognormal relaxed clock model, the piecewise linear with constant root population function and the Birth-Death speciation model, supported as the best tree prior using Bayes factor values calculated with the marginal likelihood estimates of the path sampling and stepping-stone sampling as proposed in Condamine, Nagalingum, Marshall, and Morlon (2015). We conducted four independent 100 million generation runs, sampled every 10,000 generations. Tracer v1.6 was used to check for stabilization of overall likelihood. After discarding the first 25 million generations as burn-in, the four runs were combined and summarized in a Maximum clade credibility tree (MCCT) with the LogCombiner 1.8 and TreeAnnotator 1.8 programs distributed with the BEAST package.

Bayesian concordance analysis (BCA) was conducted using BUCKy 1.4.4 (Larget et al., 2010). As BUCKy requires a single sequence to represent each marker-individual combination, we created two input files from the pruned nuclear dataset to include in the BCA two different *apo1* sequences for those specimen vouchers with more than one *apo1* copy retrieved. Thus, we included in each input file the more congruent and more conflicting *apo1* gene copy with respect to the *ndhF* phylogeny (hereafter nuclear input file a and b respectively). We created individual gene trees in MrBayes for both nuclear input files under the same model and parameters described above. Gene trees from MrBayes were used as input for the BUCKy analysis. We used the sub-program mbsum to summarize gene tree distributions generated for each locus in MrBayes and to perform BCA. The output of mbsum was subsequently used for the subprogram bucky to create a primary concordance tree with concordance factors for clades. We implemented one cold and three heated chains, using different values of $\alpha$ (0.1, 1, 5, 10, 20, 50, and 100) to model a range of prior probabilities on the number of concordance trees. We report concordance factors for the default value of 1.

To illustrate incongruences between the individual gene trees, a network graph was generated using SplitsTree version 4.14 (Huson & Bryant, 2006). For this, a filtered supernetwork was constructed from the 1,000 Bayesian posterior trees per each nuclear and plastid dataset, and filtering the splits to show only those present in a minimum of 35% input trees.

## Results

### Phylogenetic analyses of cpDNA and nucDNA datasets

Individual gene trees for the plastid DNA marker *ndhF* and the nuclear gene *apo1* are presented in Figs 1 and 2 respectively. Strict consensus tree from MP and MCCT from BI recovered similar topologies showing the same strongly supported clades, so only the BI trees are presented here for each marker, however posterior

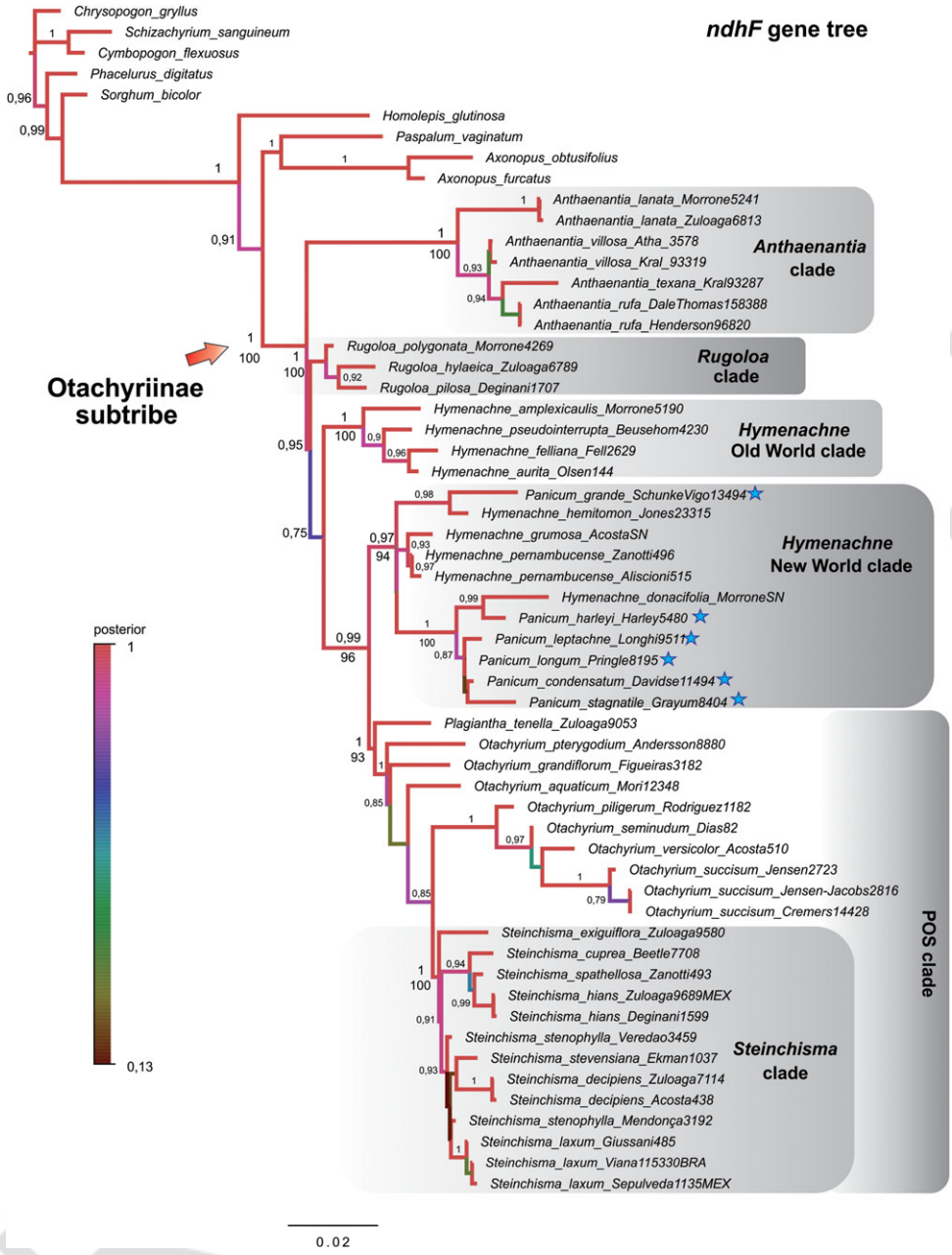


**Fig. 1.** Maximum clade credibility tree (MCCT) from 15,002 trees generated by Bayesian inference with MrBayes for the Otachyriinae subtribe using the chloroplast *ndhF* dataset. Branch colours indicate Bayesian posterior probability (PP) with red highest, brown lowest. Numbers above branches represent PP values (only PP above 0.75 are reported). Numbers below branches represent parsimony Bootstrap support (only BS values for the major clades discussed are reported). Blue stars indicate *Panicum* taxa transferred to *Hymenachne* (see taxonomic considerations section). For interpretation of the references to colour in this figure legend, the reader is referred to the electronic version of this article.

probabilities (PP) and parsimony bootstrap support (BS) values obtained under BI and MP respectively are reported.

The resulting plastid matrix alignment for 57 voucher specimens was 2068 bp long, of which 156 bp were parsimony informative. The MP analyses resulted in 20 most parsimonious trees, 291 steps long with CI = 0.62 and RI = 0.87 (Kluge, 1989; Kluge & Farris, 1969). The topology of the plastid tree (Fig. 1) agrees well with the plastid trees presented in Acosta et al. (2014). The MP and BI analyses resolved the monophyly of the Otachyriinae subtribe with strong support (PP= 1; BS= 100). The *Anthaenantia* genus is monophyletic (PP= 1; BS= 100), and resolved as sister to the remaining

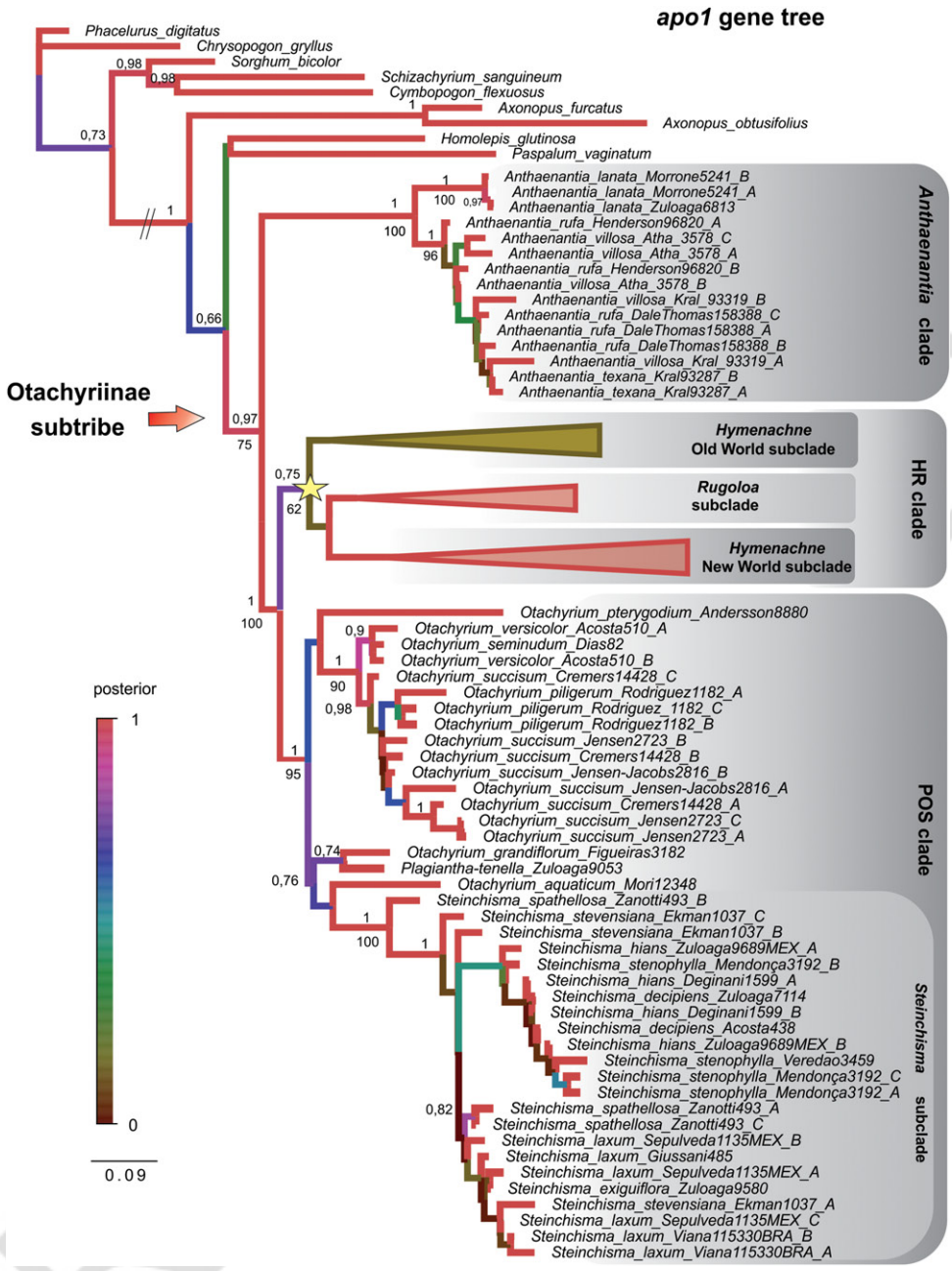


**Fig. 2.** Maximum clade credibility tree (MCCT) from 15,002 trees generated by Bayesian inference with MrBayes for the Otachyriinae subtribe using the nuclear *apo1* dataset. Branch colours indicate Bayesian posterior probability (PP) with red highest, brown lowest. Numbers above branches represent PP values (only PP above 0.75 are reported). Numbers below branches represent parsimony Bootstrap support (only BS values for the major clades discussed are reported). Letters indicate different consensus copies of the *apo1* gene for each voucher specimen. Yellow star indicates the *Hymenachne–Rugoloa* (HR) clade, with the branches leading to the subclades compressed. For interpretation of the references to colour in this figure legend, the reader is referred to the electronic version of this article.

genera of the subtribe. *Rugoloa* also was resolved as monophyletic (PP= 1; BS= 100). Regarding *Hymenachne*, this genus is non-monophyletic and two sister clades were resolved with strong support in both topologies: Old world (OW) and New world (NW) clades. The *Hymenachne* OW clade (PP= 1; BS= 100) includes the cosmopolitan *H. amplexicaulis* (Rudge) Nees along with other Old world taxa *H. aurita* (J. Presl ex Nees) Balansa, *H. felliana* (B.K. Simon) Zuloaga, and *H. pseudo-interrupta* Müll. Hal. The *Hymenachne* NW clade (PP= 0.97; BS= 94) includes the following American taxa: *H. pernambucense* (Spreng.) Zuloaga,

*H. hemitomon* (Schult.) C.C. Hsu, *H. donacifolia* (Raddi) Chase, *H. grumosa* (Nees) Zuloaga along with *Panicum incertae sedis* species: *P. leptachne*, *P. harleyi*, and *P. stagnatile*. Based on our new plastid gene tree, *P. condensatum*, *P. grande*, and *P. longum*, are included in the Otachyriinae subtribe within the *Hymenachne* NW clade. *Plagiantha*, *Otachyrium*, and *Steinchisma*, remnant genera of the subtribe, were grouped with high support (PP= 1; BS= 93). This analysis also showed that *Steinchisma* is monophyletic (PP= 1; BS= 100), and *Plagiantha* and *Otachyrium* are sisters of *Steinchisma*. The position of the genus *Otachyrium* is resolved as paraphyletic, with the genus *Steinchisma* embedded.

The dataset of the nuclear *apo1* locus was reduced to 98 sequences after the preliminary ML analysis (196 sequences). The resulting *apo1* matrix was 849 bp long, of which 169 were parsimony informative. The MP analyses resulted in 1470 most parsimonious trees, 486 steps long with CI = 0.55 and RI = 0.85 (Kluge, 1989; Kluge & Farris, 1969). In the nuclear gene tree (Fig. 2), the Otachyriinae subtribe was resolved as a highly supported monophyletic group (PP= 0.97; BS= 75).

All species of *Anthaenanthia* were grouped in a strongly supported clade (PP= 1; BS= 100) indicating that the genus is monophyletic and was resolved as the sister group of the rest of Otachyriinae species. Also, the North American species of *Anthaenantia* (i.e., *A. rufa* (Elliot) Schult., *A. villosa* (Michx.) P. Beauv., and *A. texana* Kral) were grouped as monophyletic (PP= 1; BS= 96), notwithstanding different copies being retrieved for these accessions, and were resolved as a sister group of the South American species *A. lanata* (Kunt) Benth.

The genera *Plagiantha*, *Otachyrium*, and *Steinchisma* form a highly supported clade (PP= 1; BS= 95; POS clade; Fig. 2). Within the POS clade, *Otachyrium* was resolved as paraphyletic. All sequences of *O. piligerum* Send. & Soderstr., *O. seminudum* Hack. ex Send. & Soderstr., *O. succisum* (Swallen) Send. & Soderstr. and *O. versicolor* (Döll) Henrard were grouped in a highly supported lineage (PP= 1; BS= 90), separated from the clade that groups *O. aquaticum* Send. & Soderstr., *O. grandiflorum* Send. & Soderstr. and *O. pterygodium* (Trin.) Pilg. sequences. Furthermore, the monotypic genus *Plagiantha* was entangled among the *Otachyrium* species. Additionally, sequences of species of *Steinchisma* were grouped with high support (PP= 1; BS= 100), with the exception of *Steinchisma laxum* (Sw.) Zuloaga. Sequences of *S. laxum* fell into two different clades, one containing sequences from all accessions of the rest of *Steinchisma* species (Fig. 2), whereas the second copy of *S. laxum* was grouped beside *Rugoloa* species (Fig. 3).

In the *apo1* gene tree, the *Hymenachne*, *Panicum* '*incertae sedis*', and *Rugoloa* species were grouped in a moderately supported clade (the HR clade hereafter, PP= 0.75; BS= 62; Figs 2 and 3). Within the HR clade several polyploid events may have occurred and, although relationships within the HR clade were not fully resolved, three highly supported subclades are identified: (1) the *Rugoloa* subclade (PP= 1; BS= 96), where at least one copy for all species belonging to this genus were grouped together with one genome of the polyploid *S. laxum* (described above); (2) the *Hymenachne* new world (NW) subclade (PP= 1; BS= 92), with species of *Hymenachne* and *Panicum* '*incertae sedis*' natives to America and sequences of some clones of the polyploid *H. felliana* (see below); (3) the native Old world (OW) *Hymenachne* species form a well-supported group (PP= 1; BS= 100) together with the cosmopolitan *H. amplexicaulis*, although the only *apo1* gene copy retrieved for the South American *H. donacifolia* was included in this subclade.

With regard to *Rugoloa* species, two clearly distinct *apo1* sequences were retrieved for *R. hylaeica* (Mez) Zuloaga and *R. polygonata* (Schrad.) Zuloaga; of these, one copy of both species were grouped with *R. pilosa* (Sw.) Zuloaga (called *Rugoloa* subclade hereafter). The other sequence of *R. hylaeica* and *R. polygonata* were resolved as sister to the *Hymenachne* OW subclade, within the HR clade.

The *apo1* sequence of *Panicum condensatum*, one copy of *P. grande* (*apo1* copy A) and all sequences retrieved for *P. longum* were included within the *Hymenachne* NW clade (Fig. 3). However, the *apo1* sequence copy B retrieved for *P. grande* was placed outside the *Hymenachne* NW subclade beside *Hymenachne hemitomon apo1* copy B (PP= 0.99; BS= 99). Furthermore, the *Hymenachne hemitomon* + *Panicum grande* subclade appeared twice in the tree within the HR clade: (1) in the *Hymenachne* NW subclade, and (2) sister to the *Rugoloa* subclade (although poorly supported; Fig. 3).

The different *apo1* copies retrieved for *Panicum harleyi* and *P. leptachne* were not recovered as monophyletic within the *Hymenachne* NW subclade (Fig. 3). Forcing the monophyly of *P. harleyi* and *P. leptachne apo1* sequences did not significantly decrease the likelihood (SH test, difference of log-likelihood = −4.586 and −2.581 respectively; $P > 0.05$).

The two *apo1* copies of *Hymenachne pernambucense* were recovered in different subclades inside the HR clade: (1) one copy was grouped in a distinct subclade together with *H. grumosa* (PP= 1; BS= 100) and formed a sister group of the *Rugoloa polygonata* + *R. hylaeica* + *Hymenachne* OW subclade, (2) the second

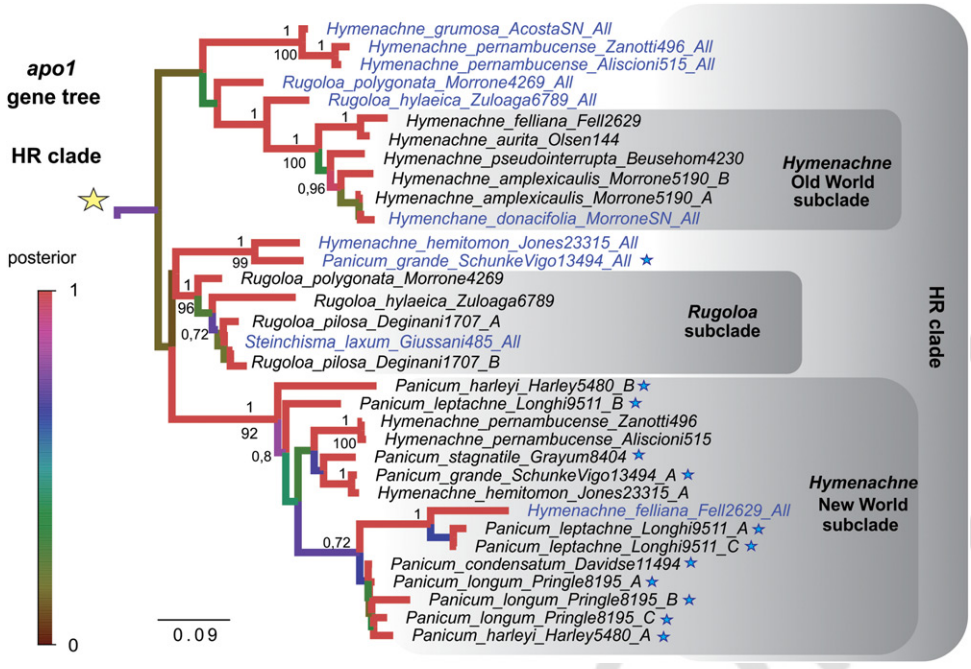


**Fig. 3.** Detailed species relationships within the *Hymenachne–Rugoloa* (HR) clade obtained with Bayesian inference for the Otachyriinae subtribe using the nuclear *apo1* dataset. Branch colours indicate Bayesian posterior probability (PP) with red highest, brown lowest. Numbers above branches represent PP values (only PP above 0.75 are reported). Numbers below branches represent parsimony Bootstrap support (only BS values for the major clades discussed are reported). Blue stars indicate *Panicum* taxa transferred to *Hymenachne* (see taxonomic considerations section). Letters indicate different consensus copies of the *apo1* gene for each voucher specimen. Blue taxa accession indicates the *apo1* gene copy considered as evidence of allopolyploidization. For interpretation of the references to colour in this figure legend, the reader is referred to the electronic version of this article.

copy was included in the *Hymenachne* NW subclade with high support (PP= 1; BS= 100).

The old world *Hymenachne* species (*H. pseudointerrupta*, *H. aurita*, and *H. felliana*), the cosmopolitan species *H. amplexicaulis* and the South American species *H. donacifolia* were grouped with high support in the *Hymenachne* OW subclade (Fig. 3). Forcing *H. donacifolia* to be included in the *Hymenachne* NW subclade in the nuclear phylogeny led to a decrease of likelihood (SH test, difference of log-likelihoods= −79.12, SD= 16.16, $P < 0.01$), as did forcing it to be sister to *H. amplexicaulis* in the chloroplast phylogeny (SH test, difference of log-likelihoods= −54.73, SD= 17.14, $P < 0.01$). Furthermore, two copies of the *apo1* were retrieved for the Australian species *H. felliana*: one copy was placed inside the *Hymenachne* OW subclade whereas the second copy fell into the *Hymenachne* NW subclade together with the South American species of *Hymenachne* and *Panicum* '*incertae sedis*' included in this study (Fig. 3).

Additionally, we tested whether we could reject monophyly of the putative polyploids (*Steinchisma laxum*, *Rugoloa hylaeica*, *R. polygonata*, *Hymenachne felliana*, *H. hemitomon*, *H. pernambucense*, and *Panicum grande*) by forcing all sequences from a given species together using SH test. Forcing the monophyly of *S. laxum*, *R. hylaeica*, *R. polygonata*, *H. felliana*, and *H. pernambucense* was significantly worse than the ML tree ($P < 0.01$), as was the monophyly of *H. hemitomon* and *P. grande* ($P < 0.05$).

Also, the cpDNA and nucDNA tree resolved *Otachyrium* as paraphyletic. However, the SH test failed to reject the hypothesis of monophyly in both chloroplast and nuclear datasets (difference of log-likelihood = −4.57 and −4.26 respectively; $P > 0.05$).

## Phylogenetic analyses of the combined datasets

The results of the coalescent-based estimation of the species tree and the primary concordance tree produced by BUCKy (hereafter BUCKy phylogeny) are presented in Fig. 4 and Fig. S1 (see supplemental material online). The BCA for both nuclear input files a and b with BUCKy retrieves a unique topology irrespective of the different a priori levels of incongruence (α varying from 0.1 to 100); also, varying the concordance prior had no effect on concordance factor (CF) values, so only the BUCKy phylogeny obtained with the nuclear input file a and *ndhF* dataset are shown (Fig. S1, see

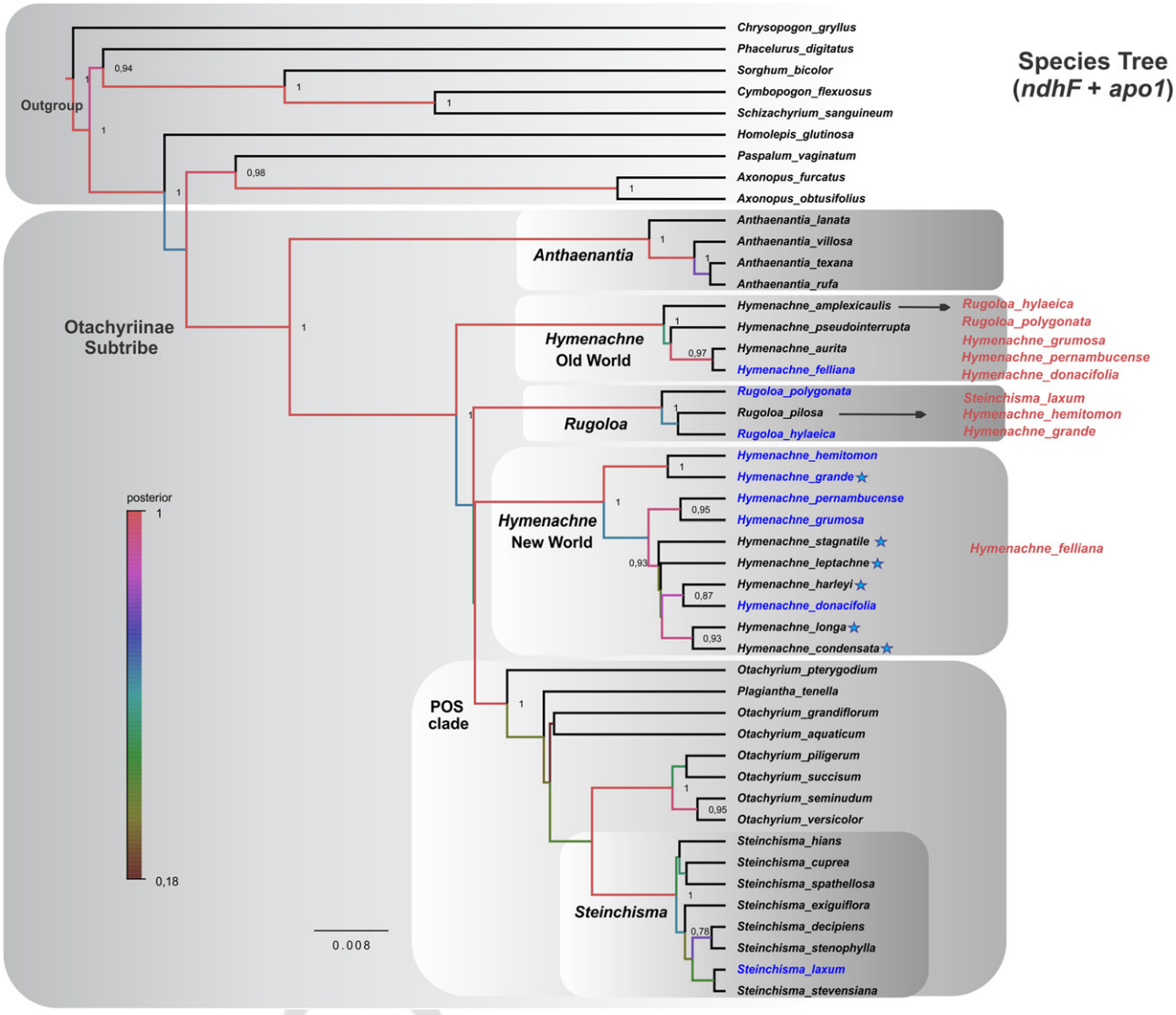


**Fig. 4.** Maximum clade credibility species tree (MCCT) estimated from nuclear (*apo1*) and chloroplast (*ndhF*) DNA regions using the multispecies coalescent method implemented in BEAST. Branch colours indicate Bayesian posterior probability (PP) with red highest, brown lowest. Numbers above branches represent PP values (only PP above 0.75 are reported). Blue stars indicate *Panicum* taxa transferred to *Hymenachne* genus (see taxonomic considerations section). Taxa in blue showed phylogenetic signature of polyploidy in the nuclear gene tree and are considered to have an allopolyploid origin. Names in red indicate the inferred position of male parental genome for allopolyploids taxa. Black arrows indicate the putative male parental diploid lineages contributing to allopolyploids. For interpretation of the references to colour in this figure legend, the reader is referred to the electronic version of this article.

supplemental material online). Both results, coalescence-based and the BCA, consistently support the monophyly of Otachyriinae (PP= 1; CF= 1 respectively). Within the Otachyriinae, several main clades were retrieved as monophyletic. First, the monophyletic *Anthaenantia* clade was resolved as the sister group of the other genera in the subtribe, grouped in a clade with PP= 1; CF= 1. Species of *Rugoloa* were recovered as monophyletic with high support values (PP= 1; CF= 0.99), and the *Hymenachne* OW clade and *Hymenachne* NW clade were both recovered as monophyletic with high PP and CF. The main sources of conflict between both trees were generated largely by the phylogenetic relationships among the *Hymenachne* OW and NW subclades and *Rugoloa*. Relationships among these three subclades were resolved in the BUCKy phylogeny as in the plastid tree but in the species tree the *Hymenachne* OW was resolved as sister to *Rugoloa*. Although the topologies differ slightly in both trees, the support values for the relationships among the affected clades are

low (PP <0.70 and CF <0.5). Another clade with high support was the clade that grouped the genera *Plagiantha*, *Otachyrium*, and *Steinchisma* (POS clade; PP= 1; CF = 1). Both trees show differences in the position of *P. tenella* Renvoize, *O. pterygodium*, *O. aquaticum*, and *O. grandiflorum* because of their low support values (PP< 0.75 and CF < 0.5). However, within this clade, the genus *Steinchisma* was recovered as monophyletic with high support in the species tree and BUCKy phylogeny (PP= 1; CF= 1).

## Taxonomic considerations

*Hymenachne bresolinii* (L.B. Sm. & Wassh.) Zuloaga, comb. nov. *Panicum bresolinii* L.B. Sm. & Wassh., Bradea 2(35): 245, Fig. 2, A–D. 1978. *Dallwatsonia bresolinii* (L.B. Sm. & Wassh.) J.R. Grande, Phytoneuron 2014(22): 3. 2014. Type: Brazil. Santa Catarina: Florianópolis, Morro Costa da Lagaô, 19 Apr 1967, *R. Klein & A. Bresolin 7360* (holotype, US-2536896; isotypes, FLOR, HBR!).

*Hymenachne condensata* (Bertol.) Chase, J. Wash. Acad. Sci. 13(9): 177. 1923. *Panicum condensatum* Bertol., Opusc. Sci. 3: 408. 1819. *Dallwatsonia condensata* (Bertol.) J.R. Grande, Phytoneuron 2014(22): 3. 2014. Type: Brazil. Rio de Janeiro: Habitat in provincia di Rio de Janeiro Brasiliae, *G. Raddi s.n.* (holotype, BOLO; isotypes, FI!, K!, PI!, US-80598!, fragment).

*Panicum auriculatum* Willd. var. *fasciculosum* Dö, in C. Martius, Fl. Bras. 2 (2): 238. 1877. *Panicum januarium* Mez, in Engler, Bot. Jahrb. Syst. 56, Beibl. 125: 4. 1921. Type: Brazil. Rio de Janeiro: Rio de Janeiro, *C. Gaudichaud 288* (isotypes, P, US 80476, W).

*Hymenachne grande* (Hitchc. & Chase) Zuloaga, comb. nov. *Panicum grande* Hitchc. & Chase, Contr. U.S. Natl. Herb. 17(6): 529, fig. 143. 1915. *Aconisia grandis* (Hitchc. & Chase) J.R. Grande, Phytoneuron 2014(22): 2. 2014. Type: Panama. Canal Zone: collected in the water of a swamp along the margin of Gatun Lake, 15 Dec 1911, *A. S. Hitchcock 9178* (holotype, US-693329!, US-693330!, US-693331!; isotypes, F!, G!, ISC!, K!, LL!, MO-848738!, NY!, P!, SI!, W!).

*Panicum myrianthum* Mez, Bot. Jahrb. Syst. 56, Beibl. 125: 3. 1921, nom. illeg. hom., not *Panicum miryanthum* Buse, 1854. Type: Suriname. Without locality, *F. W. Hostmann 434* (B!, lectotype here designated; isolectotypes, K!, US-974637!).

*Hymenachne harleyi* (Salariato, Morrone & Zuloaga) Zuloaga, comb. nov. *Panicum harleyi* Salariato, Morrone & Zuloaga, Syst. Bot. 36(1): 55, fig. 4. 2011. Type: BRAZIL. Bahia. Rio de Contas, ca. 5 km da cidade, em direção ao Pico das Almas, 13°32′58″S, 41°51′03″W, 1,107 m, 1 Aug 2006, R. M. Harley 55486 (holotype: HUEFS!).

*Hymenachne leptachne* (Döll) Zuloaga, comb. nov. *Panicum leptachne* Döll, Fl. Bras. 2(2). 195. 1877. *Dallwatsonia leptachne* (Döll) J.R. Grande, Phytoneuron 2014(22): 3. 2014. Type: Brazil. Minas Gerais. In montibus, *J.F. Widgren s.n.* (holotype, S-R-3980; isotype, S13-12967).

*Panicum pilosum* Sw. var. *polychaetum* Hackel, ErgebN. Bot. Exp. Südbras. 1: 9. 1906. TYPE. Brazil. São Paulo: prope Rio Grande inter Santos et Urbem São Paulo, 1902, 750-800 m, *M. Wacket s.n.* (holotype, W, fragment US 2907505).

*Hymenachne longa* (Hichc. & Chase) Zuloaga, comb. nov. *Panicum longum* Hitchc. & Chase, Contr. U.S. Natl. Herb. 15: 111, fig. 106. 1910. *Panicum pilosum* Sw. var. *macranthum* Scribn., Circ. Div. Agrostol. U.S.D.A. 19: 1. 1900. *Dallwatsonia longa* (Hitchc. & Chase) J.R. Grande, Phytoneuron 2014(22): 4. 2014. Type: Mexico. Veracruz: gravelly banks near Jalapa, 1250 m, 21 May 1899, *C. G. Pringle 8195* (holotype, US!; isotypes, AC 00320517!, B_10_0248978!, BM 000938687!, BR 0000006882785!, CM 0223!, ENCB 003259!, G 00099663!, 00099664!, GH 00024120!, GOET 006776!, ISC-v -0000575!, K 000309152!, MO103444!, P 00740963!, 00740964!, PH 00018682!, VR!, VT 027968!, W 19000002981!).

*Hymenachne stagnatile* (Hitchc. & Chase) Zuloaga, comb. nov. *Panicum stagnatile* Hitchc. & Chase, Contr. U.S. Natl. Herb. 17(6): 528, fig. 141. 1915. *Dallwatsonia stagnatilis* (Hitchc. & Chase) J.R. Grande, Phytoneuron 2014(22): 4. 2014. Type: Panama. Canal Zone. Frijoles, collected in water of swamp, 12 Oct 1911, *A. S. Hitchcock 8388* (holotype, US 00148027!; isotypes, BAA 00002415!, BM 000938691!, BR 0000006886684!, DAO 000465684!, F 00468896F!, G 00099802!, ISC-v-0000580!, ISC-v-0000581!, K 000309299!, LIL 000088!, LL 00370128!, MO-105081!, 105082!, NY 00381772!, MVFA 0000459!, P 00740800!, PH 000187728!, RM 0000339! W 19220009735!).

# Discussion

This study marks the first use of nuclear markers for phylogenetic analysis in tribe Paspaleae. The *apo1* gene showed enough variability to be phylogenetically informative in the species included in the present study. Also, due to the fact that this nuclear marker is a single copy in diploid species (Estep et al., 2012), it allowed us to analyse patterns of polyploidy in the Otachyriinae

subtribe, which could not be done using chloroplast markers alone. The Otachyriinae subtribe was recovered as monophyletic in all analysis with high support, although the small number of outgroups in the nuclear dataset does not make this a particularly rigorous test. Within the subtribe some major clades were identified by the *apo1* analysis, agreeing with those identified in previous studies.

Monophyly of *Anthaenantia* is supported by both the *apo1* and the *ndhF* analyses presented here. The genus is grouped with high support as sister to the other genera within the Otachyriinae. *Anthaenantia* is clearly recognized by its contracted inflorescence, with pilose, densely arranged spikelets, the lower glume absent, the upper anthecium cartilaginous, and the upper lemma not enclosing the apex of the upper palea. Phylogenetic analyses based on both plastid and nuclear genes indicate that *A. lanata*, the only South American species of *Anthaenanthia*, is sister to the rest of the North and Central American species *A. rufa*, *A. texana*, and *A. villosa*, the latter being more closely related phylogenetically. It is noteworthy to mention that *A. lanata* has been reported as a diploid (Davidse & Pohl, 1972) or tetraploid (Hidalgo et al., 2007). Hidalgo et al. (2007) suggested that, based on cytogenetics and reproductive studies, the tetraploid cytotype of *A. lanata* originated by autoduplication of the diploid cytotype. This assumption agrees with our nuclear dataset: although two copies of *A. lanata* were differentiated with high support, all copies from different accessions of this species were recovered as monophyletic, suggesting that the dominant mechanism for increased chromosome numbers in *A. lanata* is autopolyploidization.

*Rugoloa pilosa*, *R. hylaeica*, and *R. polygonata* were resolved in a clade as sister to the *Hymenachne* OW subclade in the plastid phylogeny. Chromosome counts in *Rugoloa* indicate that *R. pilosa* is diploid (Davidse & Pohl, 1978), while *R. hylaeica* and *R. polygonata* are tetraploids (Bouton et al., 1981; Pohl & Davidse, 1971). In the *apo1* phylogeny these species were grouped in two distinct subclades included within the HR clade, thus the nuclear phylogeny reveals some possible reticulation events for this genus. One copy of the *apo1* gene in the polyploids *R. hylaeica* and *R. polygonata* matched those alleles found in the diploid *R. pilosa*. However, the other copies of *R. hylaeica* and *R. polygonata* were retrieved as sister to the OW subclade of *Hymenachne*. These results support the hypothesis of an allopolyploid origin of *Rugoloa* polyploid species from genetically differentiated diploid ancestors of *Rugoloa pilosa* and some taxa related to the *Hymenachne* OW lineage.

*Hymenachne* species from the old world are grouped in the nuclear phylogeny as in the plastid phylogeny. However, it is necessary to highlight the position of *H. felliana* (two copies in different subclades) and *H. donacifolia* (only one copy retrieved but resolved in the OW subclade). Although no chromosome counts were reported for *H. felliana* both copies for this species were lumped in different subclades. Based on our results we could hypothesize an allopolyploid origin for *H. felliana*, resulting from a cross between a female and male parents related to the *Hymneachne* OW and NW clade respectively. Also, the single *apo1* copy of the South American *H. donacifolia* was included in the OW subclade. Since *H. donacifolia* has been reported as a tetraploid (Pohl & Davidse, 1971) and considering that only one accession of this species was amplified, it is quite possible that our PCR-based approach may not have uncovered all paralogues for this South American species. Combining the nuclear and plastid phylogeny, and assuming maternal chloroplast inheritance, we could infer that *H. donacifolia* is an allopolyploid, resulting from a cross between a male parent related to the cosmopolitan *H. amplexicaulis* (reported as diploid by Pohl & Davidse, 1971) and a female parent related to some species of the *Hymenachne* NW subclade, probably closely related to *P. harleyi* (as suggested by the cpDNA phylogeny). Nevertheless further support for this hypothesis is still required.

*Hymenachne grumosa* and *H. pernambucense,* native species from South America, were reported as tetraploids (Bouton et al., 1981; Núñez, 1952). Both are closely related species, sharing several exomorphological and anatomical features, and it has been argued that 'in some cases specimens are difficult to assign to one or the other species' (Zuloaga et al., 1992, p. 806). This morphological relationship between *H. pernambucense* and *H. grumosa* is supported by our results, since both species were grouped in a distinct highly supported subclade in the plastid and nuclear phylogenies. The plastid phylogeny shows *H. pernambucense* and *H. grumosa* included in the *Hymenachne* NW clade; on the other hand, both taxa are retrieved in the nuclear phylogeny in a weak supported subclade, sister to the OW subclade of *Hymenachne* within the HR clade. The incongruence between the plastid and nuclear inferred phylogenies for *H. pernambucense* and *H. grumosa* thus appears as symptomatic of incomplete lineage sorting or possible reticulate evolution. In spite of the mentioned incongruence between cpDNA and nucDNA gene trees, the second copy of the apo1 gene retrieved from *H. pernambucense* was included in the NW subclade as was the case in the cpDNA phylogeny. Therefore, it is possible that *H. pernambucense* and *H. grumosa* are species of an allopolyploid origin, a status that must be confirmed in further studies.

Molecular data showed that newly obtained sequences of *Panicum* '*incertae sedis*' species are included in the Otachyriinae subtribe. Both nuclear and chloroplast data-sets show that the new species added in the present work, *P. longum*, *P. condensatum*, and *P. grande* are related to *Hymenachne*, which is in agreement with previous morphological studies (Zuloaga et al., 1992; Zuloaga & Soderstrom, 1985). All *apo1* sequences of *P. longum* and *P. condensatum* were recovered as monophyletic and included in the *Hymenachne* NW subclade; patterns of polyploidizations were not observed for these species. In contrast, at least two different copies were found in *H. hemitomon*, *P. grande*, *P. harleyi*, and *P. leptachne*, all these taxa grouped within the NW subclade. Even though no chromosome counts are available for these species, our molecular results suggest that they might be polyploids. The SH test did not reject the monophyly of the different *apo1* copies of *P. harleyi* and *P. leptachne*, a fact that suggests that allopolyploidization is an unlikely event in these species. Nevertheless, the second copy of the *apo1* gene for *P. grande* and *H. hemitomon* were grouped as sister to the *Rugoloa* subclade. These results support the hypothesis of an allopolyploid origin for these species, with the male parent taxa related to the *Rugoloa* lineage.

*Plagiantha*, *Otachyrium*, and *Steichisma* constitute a clade in all molecular analyses. All three genera share a distinctive hardened palea and, based on this morphological synapomorphy, *Plagiantha* and *Steinchisma* were placed as synonyms of *Otachyrium* (Kellogg, 2015). On the contrary, Soreng et al. (2017) accept these tree genera as distinct taxa. Based on our *apo1* gene results, *Steinchisma* is monophyletic and derived from a paraphyletic *Otachyrium*, a result that is in agreement with the previous plastid phylogeny (Acosta et al., 2014). Although *Otachyrium* was resolved as paraphyletic, the SH test failed to reject the hypothesis of monophyly of this genus (chloroplast and nuclear dataset with $P > 0.05$). Shared ancestry of *Otachyrium* species and the distinct strongly supported clades formed by the multiple samples analysed of *Steinchisma* species in chloroplast, nuclear, and combined analyses (Figs 1, 2, and 4 respectively) reinforce their acceptance as distinct taxa. Results of combined analyses, together with the distinctive morphological features of *Otachyrium*, *Plagiantha*, and *Steinchisma* (discussed below), supports their distinction as different genera.

Most chromosome counts of species included in the POS clade are diploid (see Table S1), which is most likely the reason for no major conflicts between the nuclear and chloroplast topologies. Exceptions are *Steinchisma spathellosa* (Döll) Renvoize and *S. laxum*, reported as hexaploid (Bouton et al., 1981; Dubcovsky, Morrone, & Zuloaga, 1991) and tetraploid (Davidse & Pohl, 1978) respectively. All sequences retrieved for *S. spathellosa* were included into the *Steinchisma* clade without phylogenetic signature of allopolyploidy with other genera within Otachyriinae. The *apo1* gene tree enabled us to identify an allopolyploidization event in *S. laxum*, since this polyploid species appears in the nuclear phylogeny in two different clades. The putative maternal genome of *S. laxum* falls into the highly supported *Steinchisma* clade, as in previous chloroplast analyses (Acosta et al., 2014), while the other genome of this species is grouped together with species of *Rugoloa* within the HR clade. Zuloaga et al. (1992) found several morphological affinities among *S. laxum* (=*Panicum laxum*) and *Rugoloa* species (described as *Panicum pilosum*, *P. hylaeicum*, and *P. polygonatum*). Also, in the same work these authors stated that specimens of *S. laxum* (=*P. laxum*) have intermediate characteristics in relation to the ones present in species of *Steinchisma*. Intermediate morphological traits in allopolyploids have been observed in other grasses, such as the genus *Bouteloua* (Siqueiros-Delgado, Fisher, & Columbus, 2017). Our results indicate an allopolyploid origin for *S. laxum*, with the female parent related to the *Steinchisma* lineage and the male parent related to the *Rugoloa pilosa* lineage.

Although some levels of incongruence are apparent between both gene trees, all major conflicts were due to the presence of allopolyploid species. After these allopolyploid events were identified, the coalescent-based species tree, Bayesian concordance analysis, and the super network analysis (carried out with the pruned nuclear dataset and the cpDNA dataset), revealed that some levels of incongruence occurred mainly in the relationships among the clades comprising *Hymenachne–Rugoloa* and *incertae sedis* species of *Panicum* (Figs 3,4 and supplemental material online Fig. S1). Overall, the network analysis revealed a general tree-like divergence history of the Otachyriinae with local episodes of reticulate evolution (Fig. S2, see supplemental material online). Most relationships (- clades) identified by Acosta et al. (2014) using only plastid sequences, are found in the nuclear gene trees of this study, clades that were recovered by the filtered supernetwork and both coalescent and concordance methods.

## Implications for taxonomy and classification

Within subtribe Otachyriinae, generic relationships were well resolved by chloroplast data. Based on our results, many of those clades are supported by plastid, nuclear, and combined analyses. These include the monophyly of *Anthaenantia*, the POS clade, and the monophyly of


**Fig. 5.** Otachyriinae species. (5.1) *Otachyrium versicolor* (Acosta 510, SI), spikelets; (5.2) *Otachyrium versicolor* (Acosta 510, SI), inflorescence. (5.3) *Steinchisma hians* (Zanotti 357, SI), inflorescence. (5.4) *Steinchisma sphathellosa* (Zanotti 493, SI), spikelets. Photographs from SI (www.floraargentina.edu.ar):(5.1–5.2) by J. Acosta; (5.3–5.4) by C. Zanotti.

*Steinchisma*. Also, there is some degree of congruence between the lineages identified by the molecular analyses and the generic delimitations of earlier authors. For example *Rugoloa* and *Anthaenantia* are monophyletic in both gene trees and are here accepted as previously circumscribed.

Regarding the POS clade, *Plagiantha*, *Otachyrium*, and *Steinchisma*, Kellogg (2015) merged *Steinchisma* and *Plagiantha* into *Otachyrium*. One of the central controversies in contemporary taxonomy and systematics revolves around whether to accept or to reject paraphyletic taxa. There are false premises in the arguments for the recognition of paraphyletic taxa in botany, and it is possible that for this reason Kellogg (2015) synonymized *Steinchisma* and *Plagiantha* into *Otachyrium*, since *Otachyrium* was previously recovered as a paraphyletic taxon (Acosta et al., 2014). In the present work monophyly of *Otachyrium* was not recovered in the nuclear gene tree, species tree, and BUCKy phylogeny. Nevertheless, the SH test did not unambiguously reject the hypothesis of monophyly of the genus and *Steinchisma* is recovered as monophyletic in all analyses.

Furthermore, these genera are easily differentiated by diagnostic characters. While *Otachyrium*, *Plagiantha*, and *Steinchisma* share an expanded lower palea, species of *Otachyrium* have both glumes shorter than the spikelet, thus leaving the upper anthecium exposed, and the upper anthecium is indurate, smooth and shining, the upper lemma dark at maturity; additionally, spikelets of *Otachyrium* are solitary and arranged loosely in open and lax inflorescences (Fig. 5; Sendulsky & Soderstrom, 1984). *Plagiantha* and *Steinchisma* differ from *Otachyrium* by having the spikelets appressed on the branches of the inflorescence, with the upper glume as long as, or nearly so, the length of the spikelet, with the upper anthecium cartilaginous, covered with compound papillae that are uniformly distributed over the lemma and palea; *Plagiantha* departs from *Steinchisma* by being a $C_3$ genus, with spikelets obliquely arranged on the pedicels, and lower lemma 2–4-nerved (vs. intermediate $C_3$-$C_4$ species in *Steinchisma*, with spikelets not obliquely arranged, and lower lemma 3–5(–7) nerved). Results obtained in the molecular analyses, together with the described morphological differences, lead us to maintain both *Plagiantha* and *Steinchisma* at the generic level following the generic circumscription summarized by Acosta et al. (2014), and followed by Soreng et al. (2015, 2017).

The genus *Hymenachne* and the remnant species of *Panicum* sect. *Laxa* are in need of a re-circumscription. As suggested by the nuclear phylogeny, polyploidy played a significant role in the evolution of the Otachyriinae and phylogenetic analyses show that the history of *Hymenachne* and remnant species of '*Panicum* sect. *Laxa*' species is complex, with these taxa falling into two main subclades, the NW and OW. Although it might be feasible to split these two subclades in different genera, the absence of morphological characters to separate them makes this decision premature. Our nuclear dataset suggests that events of allopolyploidization might explain the morphological variation found in species such as *H. felliana*, *H. grumosa*, *H. pernambucense*, and *H. donacifolia*. Allopolyploids arose by fertilization of two unreduced gametes or by genome doubling after fertilization of two reduced gametes, sometimes with intermediate morphological traits (Glover, Redestig, & Dessimoz, 2016; Ramsey & Schemske, 1998). The existence of these intermediate morphological traits makes taxonomy more challenging. Based on several morphological similarities, Zuloaga and Soderstrom (1985) indicated that species of *Panicum* sect. *Laxa* could be congeneric with

*Hymenachne*. In subsequent works, several species belonging to sect. *Laxa* were transferred to *Hymenachne* based on morphological affinities and molecular results, i.e., *H. grumosa* and *H. pernambucense* (Aliscioni et al., 2003), *H. aurita*, *H. felliana*, and *H. hemitomon* (Acosta et al., 2014). Based on our molecular results, and due to the fact that *Hymenachne* and *Panicum* '*incertae sedis*' species included in the present work share several morphological characters, such as spikelets arranged in unilateral racemes, spikelets with the lower glume short, 3-nerved, upper glume and lower lemma subequal, 5-nerved, lower palea reduced or absent, lower flower absent, and upper anthecium membranous, we propose to include the remnant '*incertae sedis*' species of *Panicum* sect. *Laxa* in *Hymenachne* (for a current delimitation of *Panicum* s. str. see Zuloaga, Salariato, & Scataglini, 2018), i.e., *P. bresolinii*, *P. condensatum*, *P. harleyi*, *P. leptachne*, *P. longum*, and *P. stagnatile*.

Additionally, *Panicum grande*, a previously ungrouped $C_3$ species of *Panicum* (Aliscioni et al., 2003; Zuloaga et al., 1992) is also here treated within *Hymenachne*, based on our molecular results and morphological similarities, including spikelets arranged in congested branches, glabrous and with a lower glume 1/2 to 4/5 the spikelet length. Grande Allende (2014) placed *P. grande* in the new genus *Aconisia*, without establishing diagnostic characters for this new taxon. Similarly, this author recognized, and subsequently transferred species of *Panicum* sect. *Laxa*, to the Australian monotypic genus *Dallwatsonia*, a decision based on characters that 'may be considered diagnostic include hollow culms, second spikelets disposed in two parallel rows along the branches of the panicle, and upper anthecium pointed, membranous to more or less indurate, with conspicuous, basally thickened prickles toward the apex, and with the apex covered by the lemma' (Grande Allende, 2014, p. 4). However, these characters are present in different genera of the Otachyriinae and characters of *Dallwatsonia felliana* B.K. Simon particularly agree with the definition of *Hymenachne*; consequently *Dallwatsonia* was considered a synonym of *Hymenachne* (Acosta et al., 2014), while species transferred to this genus by Grande Allende (2014) were already placed in *Hymenachne*, i.e., *D. aurita* (J. Presl ex Nees) J.R. Grande, *D. felliana* B.K. Simon (Acosta et al., 2014), in *Rugoloa*, *D. hylaeica* (Mez) J.R. Grande, *D. pilosa* (Sw.) J.R. Grande, and *D. polygonata* (Schrad.) J.R. Grande (Acosta et al., 2014), or in the genus *Steinchisma*, i.e., *D. stevensiana* (Hitchc. & Chase) J.R. Grande (Acosta et al., 2014). Other species of *Dallwatsonia*, i.e., *D. bresolinii* (L.B. Sm. & Wassh.) J.R. Grande, *D. condensata* (Bertol.) J.R. Grande, *D. leptachne* (Döll) J.R. Grande, *D. longa* (Hitchc. & Chase) J.R. Grande, and *D. stagnatilis* (Hitchc. & Chase) J.R. Grande are classified, on the basis of molecular and morphological characters, under the genus *Hymenachne* (see taxonomic considerations below).

The present study includes the most extensive taxon sampling done so far in the subtribe Otachyriinae with data from plastid and nuclear regions that have been demonstrated to be useful in elucidating phylogenetic relationships at different taxonomic levels, clarifying the taxonomic affiliations of *Panicum* species with historically ambiguous placements. Further clarification of the nature of the reticulate evolutionary processes responsible for the complicated phylogenetic patterns will require additional studies. Our study has identified several promising topics for future studies since genetic allopolyploidy and autopolyploidy was documented using the characteristic pattern of double-labelled gene trees. Polyploidy is recognized as an important mechanism of plant diversification in grasses and could have profound effects on subsequent lineage evolution in the Otachyriinae subtribe, for instance in the evolution of photosynthetic pathways that is well documented in the group (Acosta et al., 2014). Our molecular evidence indicate that at least 40% of species within Otachyriinae show phylogenetic signature of polyploidy (16 taxa appear double-labelled in the nuclear gene trees) and support the allopolyploid origin of at least 9 taxa: *Steinchisma laxum*, *Rugoloa hylaeica* (both species reported as proto-kranz by Brown, Bouton et al., 1983; Brown, Rigsby, & Akin, 1983; Sage et al., 2014), *Rugoloa polygonata* and the $C_3$ species *Hymenachne felliana*, *H. grumosa*, *H. hemitomon*, *H. donacifolia*, *H. pernambucense*, and *P. grande*. Furthermore, our results confirm that the $C_4$ taxon *Anthaenantia* is unambiguously monophyletic and show that *Anthaenantia lanata* is an autopolyploid. Autopolyploidization might have allowed the photosynthetic diversification in other grasses subtribes, such as Neurachninae (Christin et al., 2012), and this hypothesis should not be dismissed for future studies about photosynthetic diversifications for the Otachyriinae subtribe.

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