



Morphological and genomic characterization of *Rhynchospora tenuis* complex (Cyperaceae) and its taxonomic implications

Caracterização morfológica e genômica do complexo Rhynchospora tenuis (Cyperaceae) e suas implicações taxômicas

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Abstract

Species of *Rhynchospora* sect. *Tenuis* are morphologically very similar. *Rhynchospora tenuis* complex is the most problematic species complex in this group and it concentrates entities of difficult delimitation, as is the case of *R. tenuis*, *R. tenuis* subsp. *austro-brasiliensis* and *R. enmanuelis*. Samples of these three taxonomic entities, besides *R. junciformis* and *R. breviscula* (*Dichromena*), were analyzed in a comparative way using morphologic, cytogenetic and molecular tools. Despite of high morphological similarity between these taxa, *R. tenuis* was separated from *R. tenuis* subsp. *austro-brasiliensis* and *R. enmanuelis* according to chromosome numbers ($2n = 4$ and $2n = 18$) and ISSR markers. The combined analysis of shape and size of achenes and stylopodium, number of spikelets, cytogenetic features and molecular markers suggest a clear proximity among *Rhynchospora junciformis*, *R. tenuis* subsp. *austro-brasiliensis* and *R. enmanuelis*, in relation to *R. tenuis*. These data indicate the need for a new taxonomic review of *R.* sect. *Tenuis*, mainly to solve the status and nomenclatural situation of *R. tenuis* subsp. *austro-brasiliensis* and *R. enmanuelis*.

Key words: cytotaxonomy, dysploidy, ISSR, holocentric chromosomes, Poales.

Resumo

As espécies de *Rhynchospora* sect. *Tenuis* são morfológicamente muito similares. O complexo *Rhynchospora tenuis* é o mais problemático e concentra entidades de difícil delimitação, como é o caso de *R. tenuis*, *R. tenuis* subsp. *austro-brasiliensis* e *R. enmanuelis*. Amostras dessas três entidades taxonômicas, além de *R. junciformis* e *R. breviscula* (*Dichromena*), foram analisadas de modo comparativo usando análises morfológicas, citogenéticas e moleculares. Apesar da elevada similaridade morfológica entre essas taxa, *R. tenuis* foi separada de *R. tenuis* subsp. *austro-brasiliensis* e *R. enmanuelis* de acordo com os números cromossômicos ($2n = 4$ e $2n = 18$) e marcadores ISSR. A análise combinada da forma e tamanho dos aquênios e estilopódio, número de espiguetas, características citogenéticas e de marcadores moleculares, sugere uma clara proximidade entre *Rhynchospora junciformis*, *R. tenuis* subsp. *austro-brasiliensis* e *R. enmanuelis*, em relação à *R. tenuis*. Além disso, esses dados indicam a necessidade de uma nova revisão taxonômica para *R.* sect. *Tenuis*, principalmente para resolver o status e a situação nomenclatural de *R. tenuis* subsp. *austro-brasiliensis* e *R. enmanuelis*.

Palavras-chave: citotaxonomia, disploidia, ISSR, cromossomos holocêntricos, Poales.

Introduction

Rhynchospora sect. *Tenuis* Kükenthal (Cyperaceae) is characterized by the presence of small plants with channeled to filiform leaves and achenes with transverse wrinkles and stylopodium not included in a depression of the apex of the achene (Rocha & Luceño 2002). This section

includes ca. 20 species, fourteen of these occurring in Brazil (Alves *et al.* 2009). Seventeen species were described by Kükenthal (1951), besides another three were described by Rocha & Luceño (2002). The number of taxa in this section is however doubtful due to the *Rhynchospora tenuis* complex, which may involve *Rhynchospora tenuis*

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Willd. ex Link, *R. tenuis* subsp. *austro-brasiliensis* T. Koyama, *R. enmanuelis* Luceño and Rocha, *R. riparia* (Nees) Boeck., and *R. emaciata* (Nees) Boeck. (Kükenthal 1951; Koyama 1972; Rocha & Luceño 2002). *Rhynchospora riparia* and *R. emaciata* possess striking features that distinguish them from *R. tenuis* (Vanzela *et al.* 2000; Rocha & Luceño 2002; Strong 2006). Although *R. riparia* was considered the variety *R. tenuis* var. *congesta* Kükenthal by Kükenthal (1951), and as the subspecies *R. tenuis* subsp. *riparia* (Nees) Boeck. by Koyama (1972), it is easily differentiated from *R. tenuis* due to canaliculated, larger and more rigid leaves, congested inflorescences, frequent occurrence of decumbent stalks and chromosome number $2n = 10$ (see Vanzela *et al.* 2000). *Rhynchospora emaciata* has also been considered as a synonym of *R. tenuis* (Koyama 1972, Thomas 1994), despite of evident distinct morphological features as the presence of solitary spikelets, 6–7 mm long, trigonal stalks without lateral branches, pale color achenes with an entirely marginate style base and $2n = 10$ (see Vanzela *et al.* 2000). Nevertheless, the major taxonomic confusion involves *R. tenuis*, *R. tenuis* subsp. *austro-brasiliensis* and *R. enmanuelis*, which are more similar morphologically.

Species of *Rhynchospora*, as well as other representatives of the Cyperaceae, have holocentric chromosomes (Vanzela *et al.* 1996; Vanzela & Colaço 2002), which favor the occurrence and maintenance of numeric chromosome changes, as observed in *Carex sociata* Boott (Ohkawa *et al.* 2000) and *Eleocharis maculosa* (Vahl) Roem. and Schult. (Da Silva *et al.* 2008a). Despite karyotype variations associated with symploidy and/or agmatoploidy were reported for some species as *R. tenuis* and *R. globosa* Vahl (Arguelho *et al.* 2012), polyploidy has been considered the most important event in karyotype differentiation in this genus, occurring in 59% of *Rhynchospora* species studied (Vanzela *et al.* 2000). Nevertheless, *Rhynchospora tenuis* shows the smallest chromosome number ($2n = 4$) among the Cyperaceae and plants, in general (Vanzela *et al.* 1996). The chromosome numbers were described to seven other species of the *Rhynchospora* sect. *Tenues*: *R. emaciata*, *R. riparia*, and *R. nanuzae* Luceño and Rocha with $2n = 10$, and *R. contracta* (Nees) J. Raynal and *R. enmanuelis*, *R. austro-brasiliensis* and *R. junciformis* with $2n = 18$ (Vanzela *et al.* 2000; Arguelho *et al.* 2012).

The literature points out problems in *R.* sect. *Tenues* species circumscription with some taxonomic conflicts, mainly on species close to *R. tenuis*, which presents also considerable karyotype variability. In this study we have chosen samples from three taxonomic conflicting taxa: *R. tenuis*, *R. tenuis* subsp. *austro-brasiliensis* and *R. enmanuelis*, which are very common in the southeast and southern Brazil. Besides two other species (*Rhynchospora junciformis* (Kunth) Böckeler of *R.* sect. *Tenues* and *Rhynchospora breviuscula* H. Pfeiff of *Rhynchospora* sect. *Dichromena* (Michaux) Grisebach) have been chosen for comparison using morphologic, cytogenetic and molecular analyses. The discussion considers the value of these different diagnostic characters in order to help future taxonomic revisions.

Material and Methods

Five individuals of each taxa, *Rhynchospora tenuis* (three populations), *R. tenuis* subsp. *austro-brasiliensis* (five populations), *R. enmanuelis* (two populations), *R. junciformis* (one population) and *R. breviuscula* (one population) were collected in three Brazilian states: Paraná, São Paulo, and Mato Grosso do Sul (Tab. 1). The samples of *R. enmanuelis* were obtained from Tupã, in the same locality where the type specimen was collected (Rocha & Luceño 2002), and in São Paulo, in the same locality in which Vanzela *et al.* (2000) reported chromosome number. All samples were maintained in a greenhouse at the Laboratório de Biodiversidade e Restauração de Ecossistemas (LABRE) in the Universidade Estadual de Londrina, Londrina, Paraná, Brazil.

Morphological analysis

Morphologic analysis was performed in five individuals of each species collected. In this evaluation were considered: i) size of plants, leaves and inflorescences, ii) size and shape of spikelets, achenes and stylopodium, iii) number of spikelets, anthers and axillary corymbs and iv) color of achenes. The measurements were made using graph paper, ruler, magnifying glass and stereomicroscope. The vouchers are kept in the FLOR herbarium.

Table 1 – Morphologic and karyotypic features and localities of the species of the sect. *Tenuis* and *R. breviscula*.

Morphological features	<i>R. tenuis</i>	<i>R. tenuis</i> subsp. <i>australis</i>	<i>R. emmanuelis</i>	<i>R. junceiformis</i>	<i>R. breviscula</i> (<i>Dichromena</i>)
Plant size	17 – 38 cm	16 – 40 cm	26 – 36,5 cm	24 – 41 cm	-
Leave width	0.60 - 1.45 m	0.60 - 1.50 mm	0.6 – 1.10 mm	1.0 – 1.50 mm	-
Growth habit	caespitose	caespitose	caespitose	caespitose	-
Inflorescence length	9.00 – 12.00 cm	7.50 – 14.50 cm	8.50 – 12.80 cm	9.00 – 14.00	-
Inflorescence width	3.25 – 4.55 cm	2.75 – 6.00 cm	3.50 – 4.20 cm	2.80 – 5.60	-
Number of corymbs	2	2 – 3	2 – 3	2	-
Spikelet length	3.30 – 4.10 mm	3.30 – 4.25 mm	3.40 – 4.10 mm	3.40 – 4.25 mm	-
Spikelet width	0.85 – 1.10 mm	0.70 – 1.00 mm	0.65 – 1.00 mm	0.83 – 1.10 mm	-
Number of spikelets	4 a 7	4 a 7	4 a 7	1 a 2	-
Number of stamens	3	3	3	3	-
Achene length	0.69 – 0.99 mm	1.03 – 1.14 mm	1.00 – 1.15 mm	1.07 – 1.20 mm	-
Achene width	0.66 – 0.94 mm	1.05 – 1.09 mm	0.94 – 1.08 mm	0.92 – 0.99 mm	-
Achene shape	round to obovate	obovate	obovate	rounded to oblate	-
Achene color	brown to yellowish	brown to yellowish	brown to yellowish	brown to yellowish	-
Stylododium length	0.22 – 0.25 mm	0.22 – 0.26 mm	0.25 – 0.27 mm	0.38 – 0.43 mm	-
Stylododium width	0.50 – 0.72 mm	0.87 – 0.92 mm	0.82 – 0.89 mm	0.87 – 0.91 mm	-
Stylododium shape	triangular to bilobed	triangular to bilobed	triangular to bilobed	triangular	-
Chromosome numbers	2n = 4	2n = 18	2n = 18	2n = 18	2n = 10
DSL ^a (µm)	20.98	45.66	46.21	46.37	-
LC/SC ^b (µm)	6.75 / 3.73	3.16 / 1.78	3.22 / 1.74	3.47 – 1.81	-
Localities					
Tibagi, PR	S24°34'50" - W50°15'03"				
Campo Largo, PR	S25°17'02" - W49°54'04"				
Itararé, SP	S25°28'15" - W49°40'48"				
Palmitalzinho, SP	S24°07'23" - W49°23'46"				
Tupã, SP	S24°21'58" - W49°02'69"		S21°51'47" - W50°32'81"		
São Paulo, SP			S23°33'41" - W46°43'25"		
Iporanga, SP					
Ivinhema, MS					S24°33'15" - W48°40'43"
Terenos, MS	S20°33'25" - W54°47'45"				
Anhandui, MS				S21°12'25" - W54°30'03"	

^aDSL = diploid set length and ^bLC/SC = largest chromosome/smallest chromosome.

Cytogenetic analysis

Rhynchospora tenuis, *R. tenuis* subsp. *austrobrasiliensis*, *R. enmanuelis* and *R. junciformis* were submitted to cytogenetic analyses. Somatic chromosomes were obtained from root tips, which were pretreated with 2 mM 8-hydroxyquinoline for 24 h at 10°C, fixed in absolute ethanol:glacial acetic acid (3:1, v:v) for 12 h and kept at -20°C until used, as described by Vanzela *et al.* (2000). Root tips were digested for 4 h in a mixture of 2% (v/v) cellulase and 20% (v/v) pectinase, further hydrolyzed in 1 N HCl at 60°C for 11 min, dissected in a drop of 60% acetic acid and squashed. The cover slips were removed after freezing in liquid nitrogen. The material was stained with 2% Giemsa and permanent slides mounted in Entellan. The size of the chromosomes and the length of the diploid set were determined based on five different metaphases of each species/population.

For meiotic chromosome analysis, anthers were collected and immediately fixed in absolute ethanol:glacial acetic acid (3:1, v:v) for 12 h, and then kept at -20°C until used, as described by Vanzela *et al.* (2000). Samples were hydrolyzed in 1 N HCl at 60°C for 8 min and squashed in a drop of 45% acetic acid. The coverslips were removed after freezing, as described above, and the samples were stained with 2% Giemsa. Slides were mounted with Entellan.

Fluorescent *in situ* hybridization (FISH) was performed according to Da Silva *et al.* (2008b). Slides were prepared with root tips pretreated as described above, without acid hydrolysis. The 45S rDNA probe from *Triticum aestivum* L. (pTa71; Gerlach & Bedbrook 1979) was labeled with biotin-14-dATP by nick translation and 100 ng of probe (4 µL) were mixed in a solution containing 100% formamide (15 µL), 50% polyethylene glycol (6 µL), 20 × SSC (3 µL), 100 ng of calf thymus DNA (1 µL), and 10% SDS (1 µL). The samples were denatured at 90°C for 10 min and hybridization was performed at 37°C overnight in a humidified chamber. Post-hybridization washes were carried out in 2 × SSC with 70% stringency. The probe was detected with avidin-FITC conjugate at 37 °C for 1 h and the post-detection washes were performed in 4 × SSC/0.2% Tween 20, all at room temperature. Slides were mounted with 25 µL of DABCO solution, composed of glycerol (90%), 1,4-diaza-bicyclo(2,2,2)-octane (2.3%), 20 mM Tris-HCl, pH 8.0 (2%), 2.5 mM MgCl₂ (4%) and distilled water (1.7%), with 4 µL of 2 µg/mL DAPI.

All the chromosome images were acquired in grayscale with a Leica DM4500 B microscope coupled with a DFC 300FX camera. The FISH images were overlapped with red color for DAPI and greenish-yellow (45S rDNA) for FITC, using the Leica IM50 4.0 software. The images were optimized for best contrast and brightness with Adobe Photoshop CS3 version 10.0 software.

Genomic DNA extraction and ISSR-PCR

Genomic DNA of each species, including the *R. breviscula* as outgroup, was isolated from young leaves using a CTAB extraction buffer (2% cetyltrimethyl ammonium bromide, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl, pH 8.0, 1% polyvinylpyrrolidone, and 0.2% β-mercaptoethanol) at 60°C for 1 h, and subsequently treated with 0.01% proteinase K at 60°C for 30 min, as described by Doyle and Doyle (1990), with modifications. Samples were purified with phenol/chloroform (1:1; v/v) and chloroform/isoamyl alcohol (24:1; v/v) and treated with 100 µg/mL RNase. DNA concentration was estimated using a DyNA Quant 200 (Hoefer, Inc., USA).

For ISSR-PCR (inter simple sequence repeat - polymerase chain reaction) (Zietkiewicz *et al.* 1994), the following oligomers were used as primers: TGG₅, GA₉+C, AAG₅, GTG₆, GACA₄, CAG₅, GAG₅ and TGA₆. PCR was performed in a final volume of 25 µL, containing 2.5 µL of 10× enzyme buffer, 2.0 µL of 50 mM MgCl₂, 2.0 µL of 10 mM dNTP, 1.0 µL of 5 µM primer (each), 0.2 µL of *Taq* DNA polymerase (1 U), 2.0 µL of template DNA (10 ng/µL) and 15.3 µL of ultrapure H₂O. Samples were amplified using a PTC 100 MJ Research thermal cycler, programmed for 3 min at 94°C, followed by 36 cycles of 1 min at 94°C, 30 sec at 45°C and 1 min at 72°C, with a final extension at 72°C for 5 min. To test the consistency and reproducibility of reactions, products from three different PCR reactions were submitted to electrophoresis in 1.4% Ultrapure agarose (Invitrogen™, USA) at 3 V/cm and stained with 3 µL of 10 g/mL ethidium bromide and compared. Only consistent results were used for the analysis. For analysis of ISSR polymorphisms, 5 µL of each PCR product of each sample were submitted to 9% polyacrylamide gel electrophoresis (Invitrogen™, USA) at 8 V/cm, and the bands revealed with 10% silver nitrate.

The ISSR bands were analyzed as binary characters (0 and 1) with the TREECON software - Version 1.3b. Genetic similarity data matrices were

constructed using the method of Nei and Li (1979) and group average clustering were performed by UPGMA (unweighted pair group method with arithmetic mean) with 1000 bootstrap replicates. We have included *R. breviscula* to this comparison because it belongs to *R. sect. Dichromena*, which was considered by Thomas *et al.* (2009) as a very close group of *R. sect. Tenues*. The phenogram has been constructed using data of more distant populations of *R. tenuis* and *R. tenuis* subsp. *austro-brasiliensis* in order to obtain a tree with the better resolution.

Results

The average values of the morphometric measurements of at least five fertile individuals of each taxonomic entity and population are shown in Table 1. The results showed overlap of values in most morphologic traits among all taxonomic entities. The achenes of *R. tenuis* varied significantly in size, reaching 50% size variation among populations (Fig. 1a-c). In addition, *R. tenuis* exhibited spherical/obovate achenes, while *R. tenuis* subsp. *austro-brasiliensis* and *R. enmanuelis* showed obovate ones (Fig. 1d-e, respectively). *Rhynchospora junciformis* showed spherical/oblate achenes, with size very similar to that of *R. tenuis* subsp. *austro-brasiliensis* and *R. enmanuelis* (Fig. 1f and Table 1). In general, the stylopodia were relatively similar in shape (triangular bilobed) in all taxonomic entities, except for *Rhynchospora junciformis*, which showed a triangular shape.

In general, the inflorescences exhibited two axillary corymbs, however, up to three were found in *R. tenuis* subsp. *austro-brasiliensis* and *R. enmanuelis*. The distance between corymbs varied in all species (Table 1). *Rhynchospora tenuis*, *R. tenuis* subsp. *austro-brasiliensis* and *R. enmanuelis* showed four to seven spikelets grouped, but *R. junciformis* showed one to two spikelets in the tip of culms. The spikelets of all species appeared curved when mature, but this feature was less pronounced in *R. tenuis*. All species exhibited three stamens, with some variations in size in different flowers (Fig. 1g-i). However, *R. tenuis* subsp. *austro-brasiliensis* and *R. enmanuelis* showed staminodes of different sizes in different flowers (Fig. 1i).

Cytogenetic analysis showed $2n = 4$ for *R. tenuis* (Fig. 2a) and $2n = 18$ for *R. tenuis* subsp. *austro-brasiliensis*, *R. junciformis* and *R. enmanuelis* (Fig. 2b-d, respectively), without visible karyotype variations among different populations of each species. *Rhynchospora tenuis* showed a karyotype

composed of two large and two small chromosomes and a diploid set size of about 21 μm . *Rhynchospora tenuis* subsp. *austro-brasiliensis*, *R. enmanuelis*, and *R. junciformis* displayed very similar karyotypes, with chromosomes decreasing slightly in size. The diploid set size in these three species was more than twice that found in *R. tenuis* (Tab. 1). In meiosis, just the chromosomes of *R. enmanuelis* and *R. tenuis* subsp. *austro-brasiliensis* were analyzed, pairing as $7^{\text{II}} + 1^{\text{IV}}$ (Fig. 2e-f) and only a very few cells were observed with nine bivalents. It is important to mention that some technical limitations hindered the analysis about meiosis. Due to extremely small size of anthers (a little over a millimeter) and the occurrence of some unviable anthers, with formation of staminodes, we did not get sporocytes with good quality enough to conduct a safe analysis of frequency to test the irregular meiotic configurations. In addition, because the holocentric chromosomes make possible karyotype changes resulting from symploidy and agmatoploidy and the occurrence of chromosome reduction in *R. tenuis* ($2n = 4$) and tetravalents in *R. tenuis* subsp. *austro-brasiliensis* and *R. enmanuelis*, we chose not to organize the chromosomes of these species in pairs to not incur pairing errors. The FISH with 45S rDNA probe revealed terminal signals in the two small chromosomes for *R. tenuis* (Fig. 2g). *Rhynchospora tenuis* subsp. *austro-brasiliensis*, *R. enmanuelis* (data not showed) and *R. junciformis*, showed six chromosomes with terminal hybridization signals (Figs. 2h-i), but *R. junciformis* exhibits signals in large and medium chromosomes while in the other two species the signals were in the large, medium and small chromosomes.

The eight primers used in ISSR-PCR produced fragments of 350 to 1800 bp. After the repeatability test, 116 polymorphic loci were considered and used in the genome comparison. *Rhynchospora tenuis* shared few bands with *R. tenuis* subsp. *austro-brasiliensis*, but the latter shared several bands with *R. enmanuelis* and *R. junciformis* (see an example in the Fig. 3). UPGMA grouping using the Nei & Li (1979) similarity index clearly separated *R. breviscula* from the other species, with only 9% similarity (Fig. 4). The taxa of the *R. sect. Tenues* comprised two groups, with 39% similarity. Group A consisted of two populations of *R. tenuis*, with 77% of similarity. Group B, which comprised *R. tenuis* subsp. *austro-brasiliensis*, *R. enmanuelis* and *R. junciformis*, was divided into three subgroups, both with 60% similarity. The first subgroup, named B1, includes only one population of *R. junciformis*, and the second or B2, grouped *R. tenuis* subsp. *austro-brasiliensis*

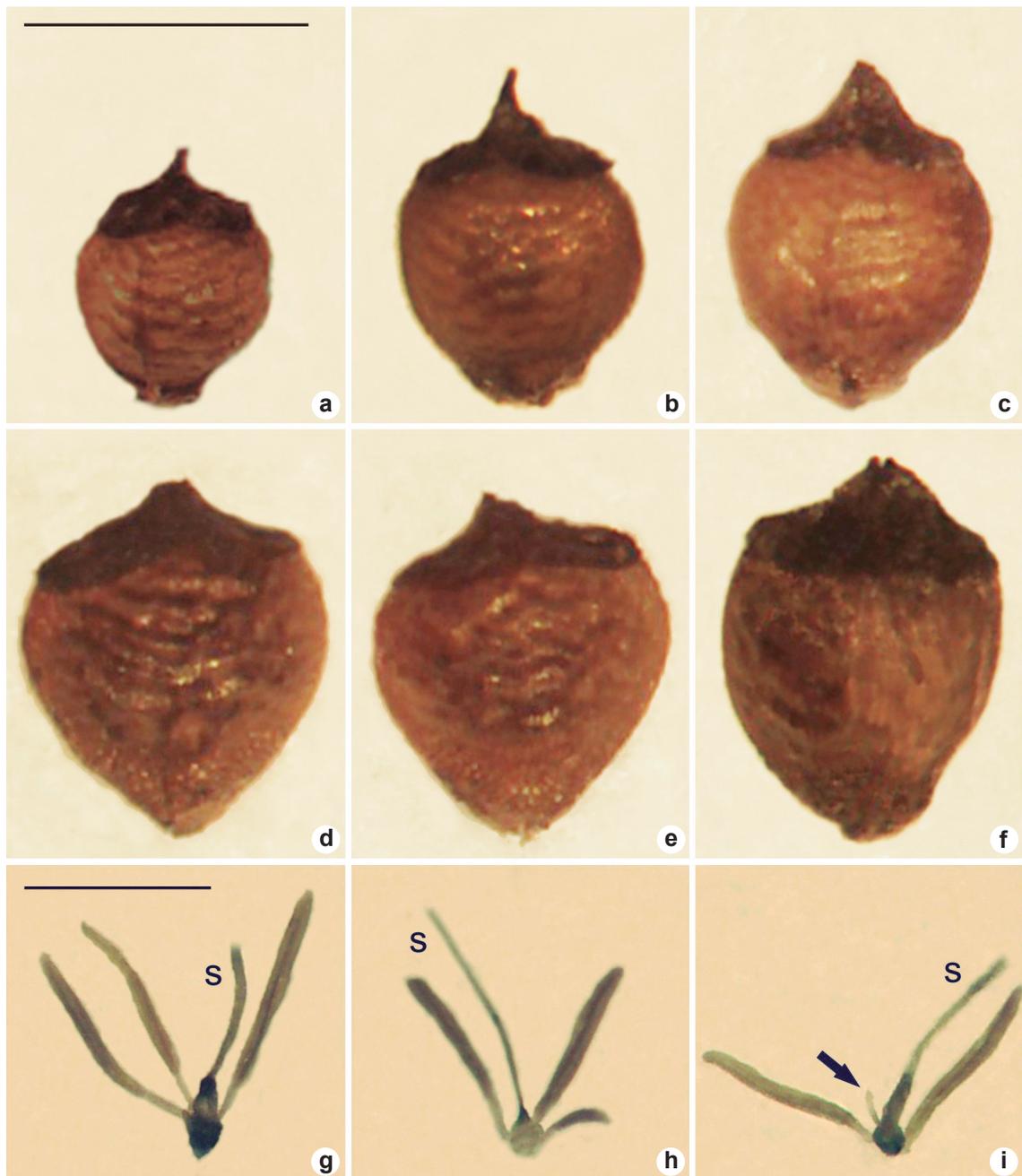


Figure 1 – Fruit and flower morphology of Brazilian species of *Rhynchospora* section *Tenuis* – a-f. image of the achenes; a-c. achenes of *Rhynchospora tenuis*. Note the evident variation in the size of achenes from different samples. d. achene of *R. tenuis* subsp. *austro-brasiliensis*; e. achene of *R. enmanuelis*. Note the similarity between achenes of d and e-f. achene of *R. junciformis*. Note the wider stylopodium in relation to other species. g-i. anthers of *R. enmanuelis*. Note the existence of three anthers of similar size (g), two larger and one smaller (h) and little staminodes indicated by arrow (i). S = style. Scale bars = 1 mm.

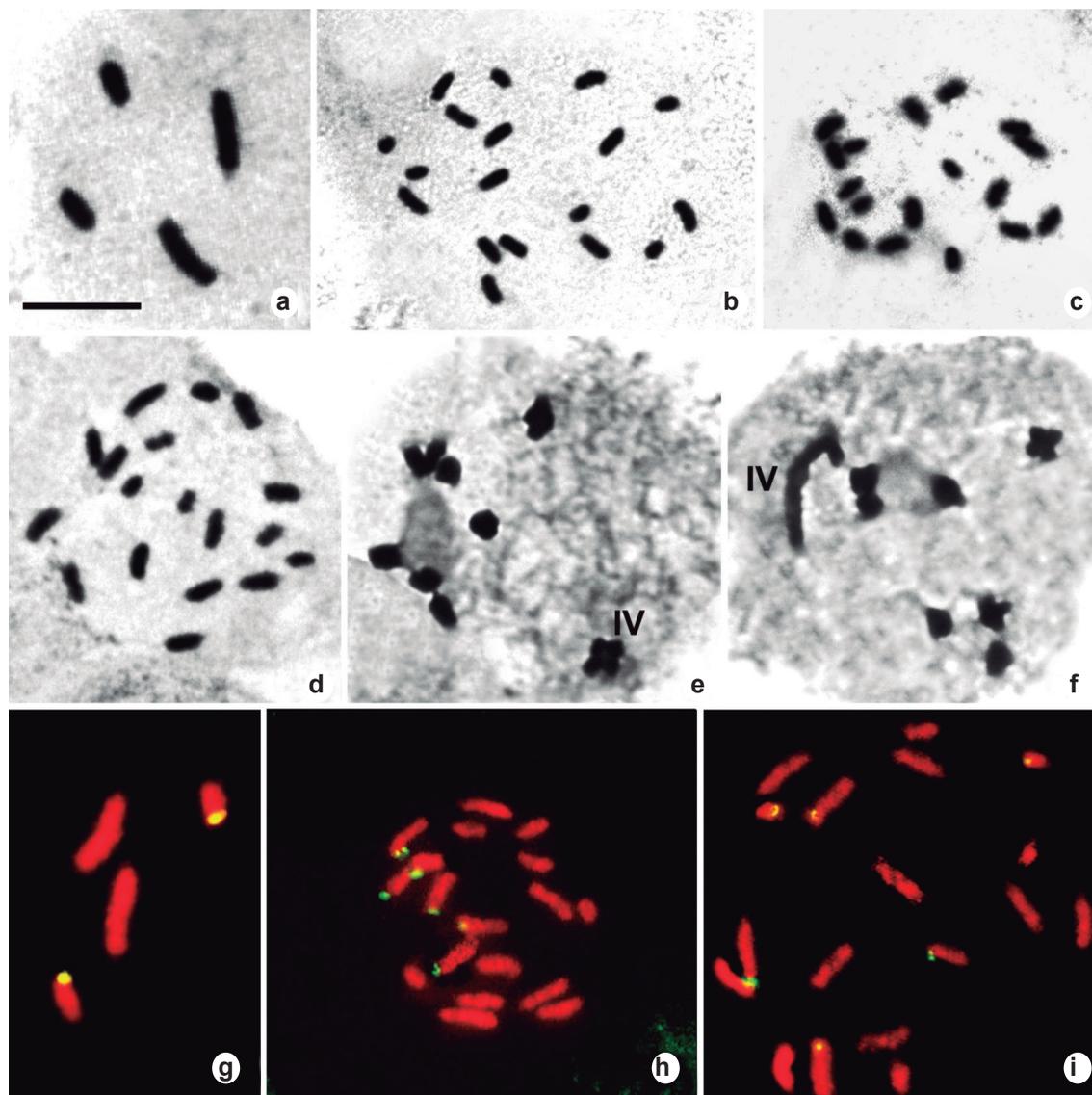


Figure 2 – Chromosomes of Brazilian species of *Rhynchospora* section *Tenuis* – a. mitotic chromosomes of *Rhynchospora tenuis* with $2n = 4$; b. *R. tenuis* subsp. *austro-brasiliensis* with $2n = 18$; c. *R. junciformis* with $2n = 18$; d. *R. enmanuelis* with $2n = 18$. e-f. meiotic chromosomes of *R. tenuis* subsp. *austro-brasiliensis* and *R. enmanuelis* with 7^{II} and 1^{IV} . Numbers indicate tetraivalent configurations; g. FISH with 45S rDNA probe in mitotic chromosomes of *R. tenuis*; h. *R. junciformis*; i. *R. tenuis* subsp. *austro-brasiliensis*. Bar = 10 μm .

and *R. enmanuelis* with at least 68% similarity. In group C, one population of *R. tenuis* subsp. *austro-brasiliensis* (Itararé, SP) showed 85% similarity with *R. enmanuelis* from São Paulo, SP (Fig. 4).

Discussion

Rhynchospora tenuis, *R. tenuis* subsp. *austro-brasiliensis* and *R. enmanuelis* are easily confused in the field, mainly because the features used in diagnosis overlap in size and shape. Our analysis

also showed overlapping of the values for all morphologic characters, including *R. junciformis*, except for shape of stylopodium in the last one. When our data on morphology were compared with the morphological features reported by Barros (1960), Koyama (1972), Rocha & Luceño (2002) and Strong (2006) all these species were found to share similar features. However, when our results on size and shape of achenes and stylopodium, number of terminal spikelets, karyotypic features and ISSR

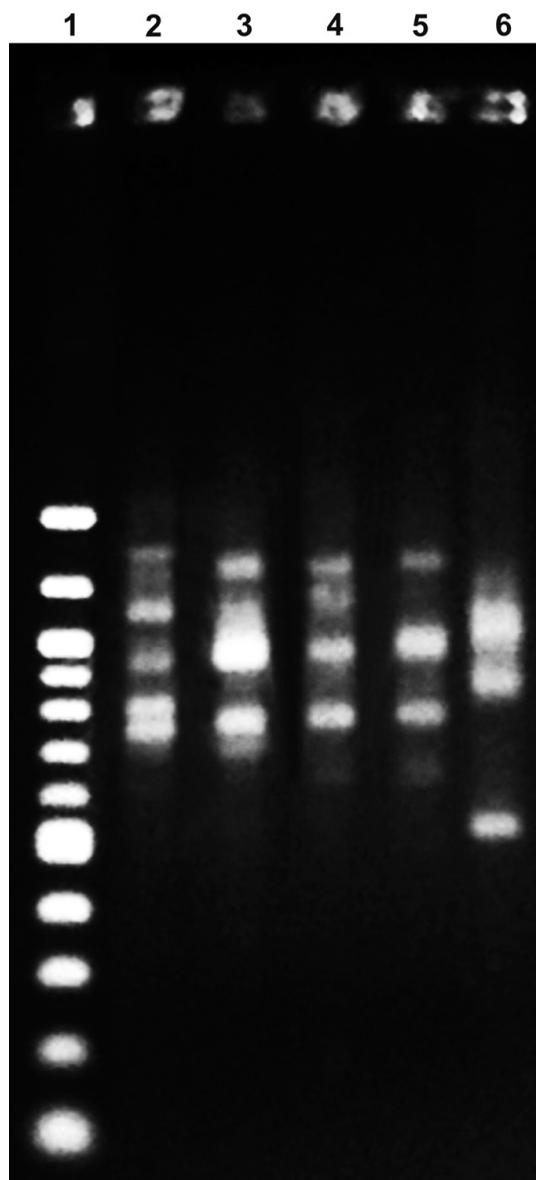


Figure 3 – Agarose gel demonstrating the repeatability tests, which shows the bands obtained after ISSR-PCR with the GA₉+C primer. 1) 100 bp, 2) *R. tenuis*, 3) *R. tenuis* subsp. *austro-brasiliensis*, 4) *R. enmanuelis*, 5) *R. junciformis* and 6) *R. breviuscula*. Note the similarities between *R. junciformis* and *R. tenuis* subsp. *austro-brasiliensis*, including *R. enmanuelis*, and the differences in relation to *R. tenuis*.

markers were jointly analyzed and compared, they point to new possibilities in respect to the resolution of species/subspecies associated with *R. tenuis*, and reinforce the need for evaluations using different macro and micromorphological features to enhance the taxonomic analysis in this genus.

The literature shows that *Rhynchospora junciformis* can be differentiated from other species of *R. sect. Tenuis* based on the smaller number of spikelets in the tip of culms, morphology of achenes and stylopodium (Barros 1960; Rocha & Luceño 2002, Strong 2006). Samples collected in Anhanduí exhibited overlap of some morphological features, however this population was differentiated from the other taxa by number of spikelets (1–2), achene shape (rounded to oblate) and stylopodium length (0.38–0.43 mm). These samples exhibited triangular bilobed shape of stylopodium, which reach one-third of the achene length, being very similar to *R. junciformis* var. *monocarpa* Kük. described by Barros (1960). However, these were very different from the description of Rocha & Luceño (2002) and Strong (2006). In this last case the stylopodium appeared to be reduced to a mucro. The karyotype of *R. junciformis* with $2n = 18$ is very similar to that of *R. tenuis* subsp. *austro-brasiliensis* and *R. enmanuelis* (previously described by Vanzela *et al.* 2000; Arguelho *et al.* 2012). The similarities between these three species were also revealed after analysis with ISSR markers, suggesting that *R. junciformis* is relatively well-defined in terms of morphology and seems to be closer to *R. tenuis* subsp. *austro-brasiliensis* and *R. enmanuelis* than to *R. tenuis*.

Rhynchospora enmanuelis is morphologically very similar to *R. tenuis* subsp. *austro-brasiliensis*. Rocha & Luceño (2002) used measurements of leaves, length of the culms, spikelets and stylopodium, number of axillary corymbs (2–3) and, especially, the presence of two stamens, to describe *R. enmanuelis* as a new species. Except for the occurrence of two stamens, all other characteristics were previously described for *R. tenuis* subsp. *austro-brasiliensis* (Koyama 1972; Strong 2006). Our results showed both *R. enmanuelis* and *R. tenuis* subsp. *austro-brasiliensis* presenting three stamens, as well as other species of the *Rhynchospora tenuis* complex (Strong 2006). It is important to point out that the samples of *R. enmanuelis* studied here were collected in the exact same localities of the type specimen, which were studied by Rocha & Luceño (2002), and that samples of *R. tenuis* subsp. *austro-brasiliensis* were obtained in the common occurrence area described by Koyama (1972). Another important difference observed here is the presence of short staminodes in several spikelets in both *R. enmanuelis* and *R. tenuis* subsp. *austro-brasiliensis*. These structures

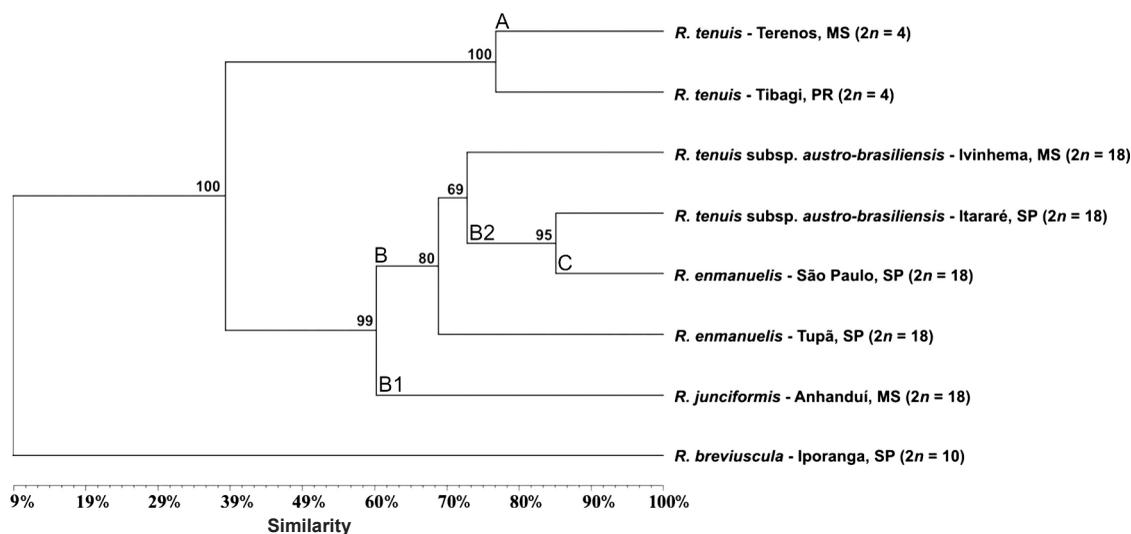


Figure 4 – Phenogram of *Rhynchospora tenuis* complex. The bottom bar refers to Nei & Li (1979) genetic similarity. All populations were analyzed. Observe that *R. tenuis* subsp. *austro-brasiliensis* and *R. enmanuelis* are grouped together, closer to *R. junciformis* than *R. tenuis*. Numbers refer to bootstrap values.

had not been mentioned for species of the *R.* sect. *Tenues* in the reviews of Barros (1960), Koyama (1972), Rocha & Luceño (2002) and Strong (2006).

Chromosome analysis revealed that *R. enmanuelis* and *R. tenuis* subsp. *austro-brasiliensis* possess very similar karyotypes, with $2n = 18$ and the 45S rDNA in the same chromosome pairs and position. Similarities were also observed after analysis of ISSR markers. The phenogram grouped these two species always in the same clade. The set of information obtained from morphologic, cytogenetic and molecular analyses strongly suggest a deep relationship between these two taxonomic entities.

Koyama (1972) used some features such as anther size, length and shape (curved when mature) of spikelets, and the distance between the corymbs in the culms to define *R. tenuis* subsp. *austro-brasiliensis* as a subspecies of *R. tenuis*. Our morphologic analysis also showed overlap of several values and characters between *R. tenuis* and *R. tenuis* subsp. *austro-brasiliensis*, except for subtle differences in the shape and size of achenes. Despite morphological features do not give support to consider *R. tenuis* subsp. *austro-brasiliensis* as a new species, the information obtained from ISSR markers and karyotypes revealed that there are strong genetic differences between these two entities. The ISSR analysis separated *R. tenuis*

from *R. tenuis* subsp. *austro-brasiliensis*, which exhibited a similarity of only 39%. Interestingly, *R. tenuis* subsp. *austro-brasiliensis* and *R. junciformis*, both with $2n = 18$, showed 60% genetic similarity, suggesting that these two species are distant from *R. tenuis* with $2n = 4$. It is important to mention that intermediate chromosome numbers between *R. tenuis* and *R. tenuis* subsp. *austro-brasiliensis*, even in sympatric conditions, were not reported until date.

The occurrence of $7^{II} + 1^{IV}$ in some samples of *R. tenuis* subsp. *austro-brasiliensis* indicate a possible polyploid origin for $2n = 18$, associated with dysploidy events. This explanation seems reasonable if we consider the basic number $x = 5$ of Cyperaceae (Löve *et al.* 1957) and the existence of karyotypes with $2n = 10$ in other species of the *R.* sect. *Tenues*, namely *R. nanuzae* Luceño and Rocha, *R. riparia* and *R. emaciata* (Vanzela *et al.* 2000). The association of numeric and structural changes in the karyotype differentiation of Cyperaceae were well illustrated in studies within *Rhynchospora* (Vanzela *et al.* 1996; Luceño *et al.* 1998; Vanzela *et al.* 2000; Arguelho *et al.* 2012) and *Eleocharis* (Da Silva *et al.* 2008a, b, 2010).

The combined use of morphologic, cytogenetic and molecular techniques produced sufficient information to point out new paths toward the resolution of taxonomic problems in the *R.* sect. *Tenues*. Although we know the necessity of a broader

analysis, including a larger number of populations and important species such as *Rhynchospora nanuzae*, *R. riparia* and *R. emaciata* in order to improve the taxonomic resolution in this group, our results have been effective to show the high similarity between *R. enmanuelis* and *R. tenuis* subsp. *austro-brasiliensis* in relation to other species of the sect. *Tenues*. The results reinforce also the need of a new taxonomic revision of the *Rhynchospora* sect. *Tenues*, especially in the nomenclatural status of *R. tenuis* subsp. *austro-brasiliensis* and *R. enmanuelis*.

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