

# **Estudo citogenético e evolutivo em espécies brasileiras de *Eleocharis* (Cyperaceae)**

Tese apresentada ao curso de  
pós-graduação em Genética  
para obtenção do Título de  
Doutor em Genética, da  
Universidade Estadual Paulista

Orientador: Prof. Dr. André Luis Laforga Vanzela

2010

Carlos Roberto Maximiano da Silva

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2010

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A minha noiva Renata da Rosa, em especial, por tornar meus sonhos realidades.

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## Resumo

A família Cyperaceae é a terceira maior entre as monocotiledôneas, com 42 gêneros encontrados no Brasil. *Eleocharis* é o quarto gênero mais diverso, com 69 espécies encontradas em áreas alagadas, margens de rios e lagos. São plantas com morfologia simples, porém, com uma grande variação morfológica. Isto tem dificultado a identificação e organização taxonômica deste grupo. Este gênero, assim como toda a família, também é conhecido por possuir cromossomos holocêntricos, meiose pós-reducional e formação de pseudomônades, além de uma grande variação intra- e interespecífica no número cromossômico. Buscando compreender a evolução cariotípica e esclarecer problemas taxonômicos neste grupo, foram estudadas 259 amostras de 10 estados brasileiros, representando cerca de 40% das espécies. Análises citogenéticas revelaram variação cromossômica interespecífica de  $2n = 6$  (*E. subarticulata* e *E. maculosa*) até  $2n = 60$  (*E. laeviglumis*), causada principalmente por poliploidia. Apesar desta variação, o número básico  $x = 5$  é sugerido. As maiores variações intraespecíficas foram encontradas em *E. maculosa* ( $2n=10, 8, 7$  e  $6$ ) decorrente de simploidia e no complexo de espécies formado por *E. viridans* e *E. niederleinii*, nas quais os dados citogenéticos e moleculares indicam uma origem híbrida associada à fissão e/ou fusão cromossômicas e poliploidia. A localização física dos sítios de DNAr 45S e 5S por FISH mostrou sinais de 45S sempre terminais e com múltiplos sítios (2 a 10). Esta multiplicação de sítios pode ser resultado de amplificação seguido de dispersão pelas pontas dos cromossomos com mesmo tamanho. O sítio de DNAr 5S foi menos variável em número (2 a 4) por cariótipos, contudo, variou entre as posições terminais e intersticiais. Este estudo mostra que os mecanismos responsáveis pela diversidade cariotípica em *Eleocharis* são próximos àqueles reportados para o gênero *Rhynchospora* e *Carex*, com diferenças na predominância de agmatoploidia e simploidia em *Carex* e poliploidia nos demais grupos. Em termos taxonômicos, este estudo reforçou ainda mais a grande diversidade dentro do subgênero *Eleocharis*, bem como a separação de *Limnochloa* dos demais subgêneros, como proposto em estudos de filogenia molecular.

**Palavras chave:** Citotaxonomia, Cromossomos holocêntricos, Poliploidia, Rearranjo cromossômico

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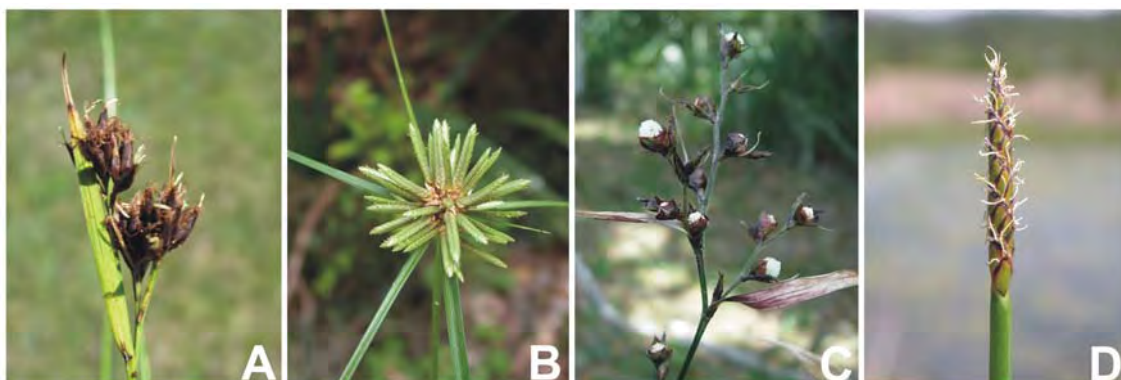
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## 1. Introdução

### 1.1. Família Cyperaceae

Cyperaceae Juss. é a terceira maior família das monocotiledôneas e apresenta distribuição cosmopolita. Seus representantes ocorrem em uma variedade de habitats, sendo mais frequentes em áreas úmidas como brejos, pântanos, margens de rios, charcos e ambientes de restinga (Dahlgren *et al.* 1985, Goetghebeur 1998 e Judd *et al.* 1999).

Esta família possui mais de 5.500 espécies, distribuídas em mais de 109 gêneros (Govaerts *et al.* 2007). O primeiro levantamento da flora de Cyperaceae realizado para todo o Brasil registrou cerca de 300 espécies em 65 gêneros (Ness 1842). Recentemente, foram identificadas 678 espécies em 42 gêneros, sendo que os de maior riqueza de espécies são *Rhynchospora* Vahl (157 espécies), *Cyperus* L. (101 espécies), *Scleria* Berg. (82 espécies) e *Eleocharis* R. Br., com 69 espécies (Alves *et al.* 2009). Algumas imagens de representantes desses grupos são apresentadas na Figura 1.



**Figura 1.** Diversidade da inflorescência das espécies de Cyperaceae dos quatro gêneros mais abundantes no Brasil. A) *Rhynchospora polyantha* Steud.; B) *Cyperus incommutus* Kunth; C) *Scleria panicoides* Kunth e D) *Eleocharis laevigulmis* R. Trevisan & Boldrini

Estudos filogenéticos, realizados por Muasya *et al.* (2000), sugerem que esta família compreende um grupo monofilético, próximo de Juncaceae. No entanto, a determinação das relações taxonômicas em Cyperaceae pode ser considerada difícil, devido ao tamanho reduzido das flores e às inflorescências condensadas. Segundo o estudo taxonômico realizado por Bruhl (1995), a família é dividida em duas subfamílias (Cyperoideae e Caricoideae) compostas por 14 tribos. Goetghebeur (1998) concordou com as 14 tribos, no entanto, este autor dividiu a família em quatro subfamílias (Cyperoideae, Caricoideae, Scleroioideae e Mapanoideae). Com o auxílio de dados moleculares, Simpson *et al.* (2003, 2007) e Muasya *et al.* (2009) dividiram a família Cyperaceae em duas subfamílias: Cyperoideae e Mapanoideae, composta pelas mesmas 14 tribos. Apesar das diferentes classificações, o gênero *Eleocharis* foi sempre incluído à tribo Eleocharidae, subfamília Cyperoideae.

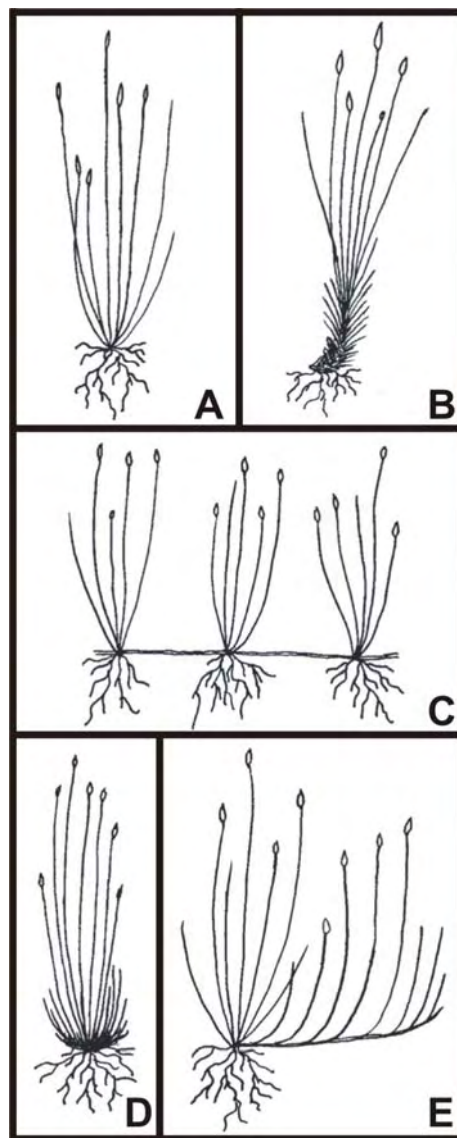
## **1.2. Características morfológicas do gênero *Eleocharis***

*Eleocharis* possui aproximadamente 250 espécies, das quais 16 são endêmicas do Brasil (Alves *et al.* 2009). Apesar de ser um gênero cosmopolita, a maioria das espécies está nas regiões tropicais e subtropicais das Américas (Govaerts *et al.* 2007). São encontradas em solos que ficam temporariamente úmidos ou inundados, e algumas espécies vivem total ou parcialmente submersas (Trevisan e Boldrini 2008). Segundo Svenson (1929), *Eleocharis* é um gênero natural que fazia parte do gênero *Scirpus* L.. Atualmente, *Eleocharis* encontra-se separado de *Scirpus* devido à ocorrência de inflorescência única sem brácteas involucrais e estilopódio persistente, endurecido, dilatado e articulado com o ovário. *Scirpus*, de acordo com Goetghebeur (1998), possui inflorescência composta com brácteas involucrais, estilopódio pouco nítido e não espessado.



As espécies do gênero *Eleocharis* possuem: colmo fotossintetizante com bainhas na base, glumas geralmente imbricadas e espiraladas ao longo do eixo da espiguetas, flores bissexuais, perigônio reduzido a cerdas ou ausente, estames geralmente trífidos e estilete 2-3-fido (Svenson 1929 e González-Elizondo 1994). Segundo Svenson (1929, 1932, 1934, 1937 e 1939), as espécies podem ter hábitos cespitoso, rizomatoso ou estolonífero. Contudo, Trevisan e Boldrini (2008) organizaram as espécies brasileiras em cespitosas com e sem formação de cáudice; cespitosas-estoloníferas com estolhos de entrenós longos; cespitosas-rizomatosas com rizomas curtos e cespitosas-rizomatosas com rizomas alongados de entrenós curtos. A Figura 2 mostra os esquemas com os hábitos propostos por estes autores.

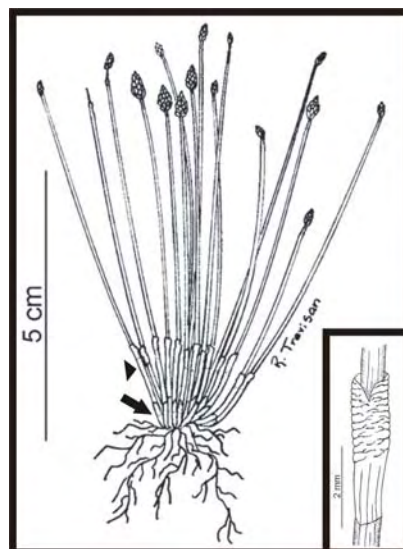
Os caules das ciperáceas, também chamados de colmos, são geralmente sólidos, frequentemente 3-angulado, podendo ser ramificados ou não (Mohlenbrock 1976). Em *Eleocharis*, o colmo é um escape, o qual em secção transversal pode apresentar três ou mais ângulos, ou ainda ser redondo a elíptico, e com função fotossintetizante (Ueno *et al.* 1989). Cada colmo ereto que sai do rizoma de *Eleocharis* é um ramo terminal que possui duas bainhas. Uma inferior que é frouxa, membranácea e mais fendida, enquanto que a superior é cilíndrica e envolve o



**Figura 2.** Esquema mostrando os hábitos em *Eleocharis*. A) Cespitoso; B) Cespitoso, com formação de cáudice; C) Cespitoso-estolonífero, com estolhos de entrenós longos; D) Cespitoso-rizomatoso com rizomas curtos e E) Cespitoso-rizomatoso com rizomas alongados de entrenós curtos. Fonte: Trevisan e Boldrini (2008).

colmo (veja a Figura 3). Esta última estrutura tem valor taxonômico, sobretudo o ápice. A presença ou ausência de múcron dorsal, o ápice truncado ou oblíquo, o apêndice hialino, rugoso ou não, são alguns dos principais caracteres diagnósticos das bainhas.

As inflorescências das Cyperaceae são compostas por uma ou mais brácteas involucrais. *Eleocharis*, no entanto, não possuem esses invólucros, sendo isto uma das características que definem o gênero. As inflorescências são as mais simplificadas dentro da família, aparecendo como

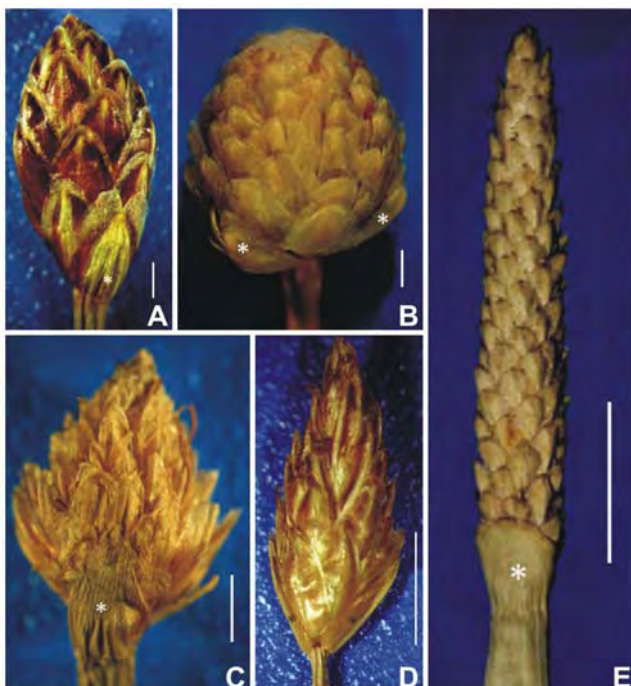


**Figura 3:** Desenho de *E. flavescens*. A seta aponta para a bainha inferior. A cabeça de seta aponta para a bainha superior. Em maior aumento, o detalhe do ápice hialino rugoso hialino da bainha superior. Fonte: Trevisan e Boldrini (2008).

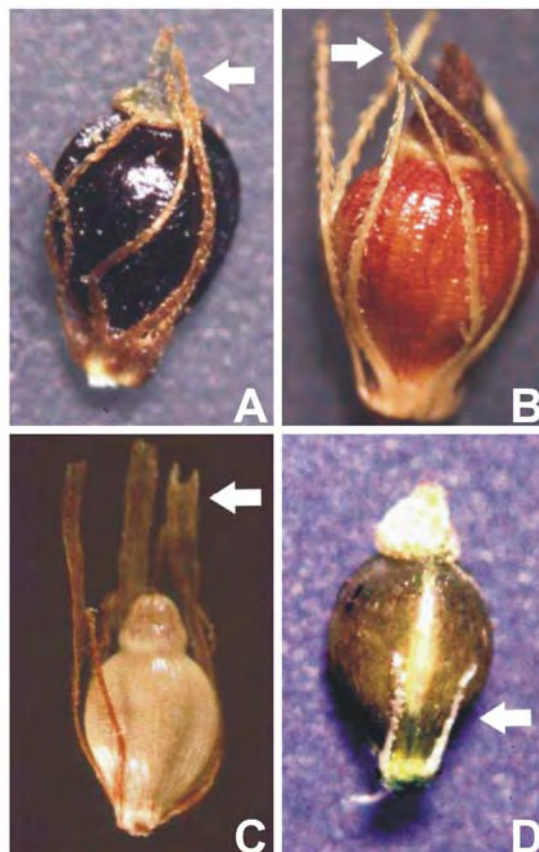
espigueta única no ápice do colmo (veja a Figura 4). A polinização é anemófila, como na maioria das Cyperaceae. As espécies apresentam redução das características de atração a visitantes. Por outro lado, produzem grande quantidade de grãos de pólen.

O número de estames em plantas anemófilas é geralmente grande, e as anteras encontram-se frequentemente expostas, elevadas para fora da flor por filetes longos e flexíveis, com pedicelo móvel. O estigma é geralmente plumoso e grande, ficando exposto para aumentar a probabilidade de contato com os grãos de pólen. Os aquênios apresentam as características morfológicas mais significativas na diferenciação das espécies de *Eleocharis* (Svenson 1929, 1939 e González-Elizondo e Peterson 1997). As mais importantes são a forma e a coloração da base do estilete, bem como o tamanho e o número de cerdas periânticas. Neste gênero, assim como em toda a família, algumas espécies possuem aquênios com superfícies lisas e outras com superfície ornamentada (Trevisan e Boldrini 2008) (veja a Figura 5). Contudo, quando observados ao

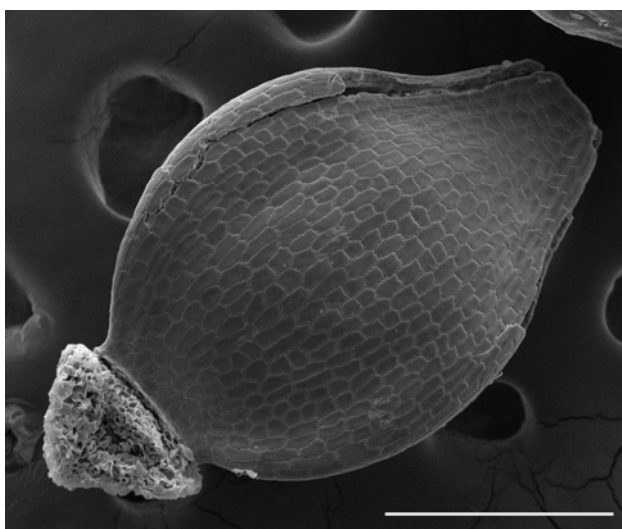
microscópio eletrônico de varredura, todos os aquênios apresentam ornamentação com valor taxonômico (Menapace 1990 e Lye 2000) (veja a Figura 6).



**Figura 4.** Diferentes formas de espiguetas em *Eleocharis*. A) Elipsóide em *E. maculosa*. B) Globosa de *E. geniculata*. C) Obovóide de *E. pachystyla*. D) Elipsóide de *E. sellowiana*. A-D) Barra = 1mm. E) Cilíndrica em *E. interstincta*. Barra = 10 mm. Os asteriscos indicam as basais. Fonte: Gil e Bove (2007)



**Figura 5:** Aquênios de *Eleocharis*. A) Com superfície lisa em *E. debilis*. A seta aponta a cerda periântica com o mesmo tamanho do corpo do aquênio. B) Com superfície ornamentada em *E. interstincta*. A seta aponta a cerda periântica maior que o comprimento do corpo do aquênio. C) com superfície ornamentada em *E. mutata*. A seta aponta a cerda periântica muito maior que o corpo do aquênio. D) Com superfície lisa de *E. minima*. A seta aponta a cerda periântica menor que o comprimento do aquênio. Observe ainda as diferentes formas e cores dos frutos.

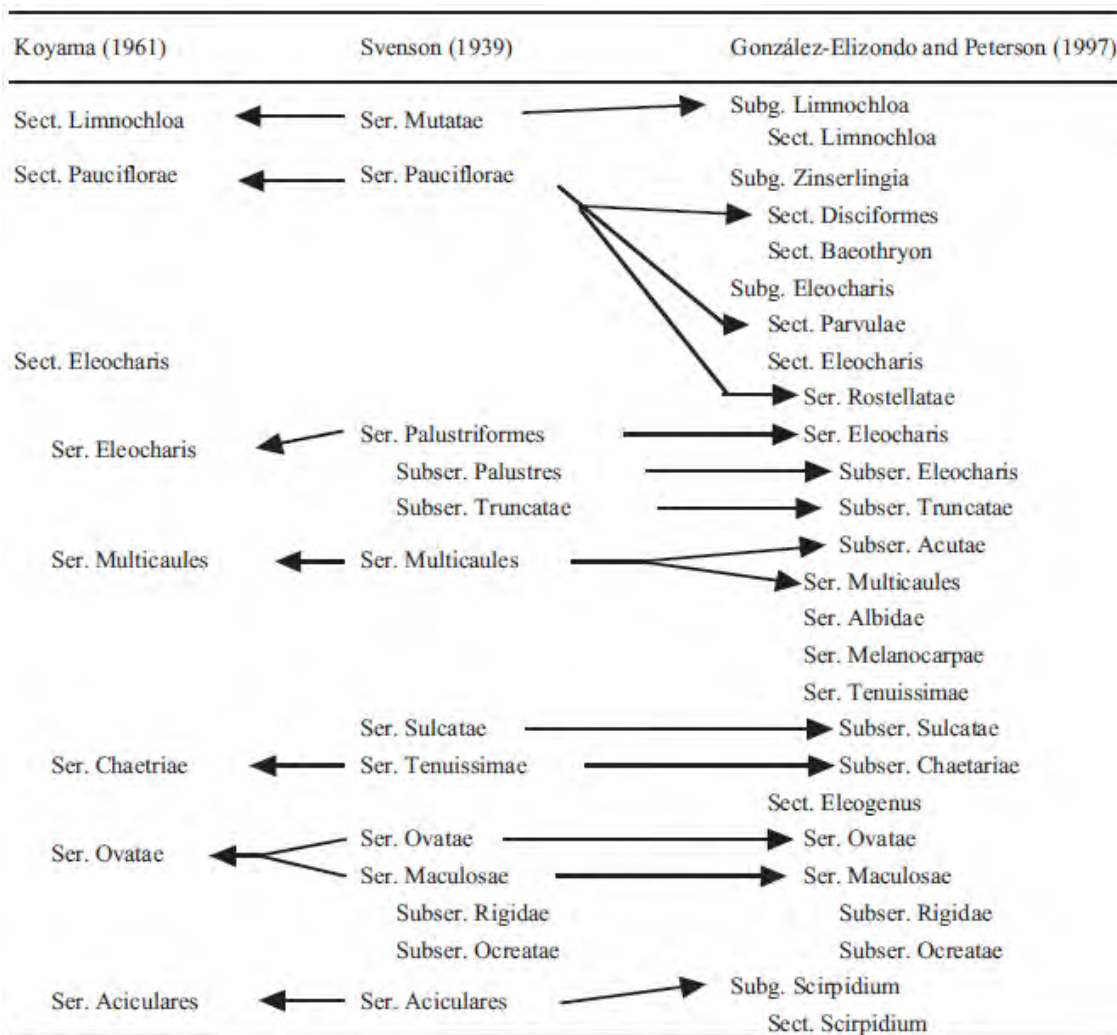


**Figura 6:** Aquênio de *Eleocharis minima* em microscopia eletrônica de varredura, mostrando ornamentação em sua superfície. Barra = 300µm

### 1.3. Relações taxonômicas e filogenéticas do gênero *Eleocharis*

*Eleocharis* é um grupo marcado por morfologia simples, mas com grande variação intraespecífica (Simpson 1988). Isto tem proporcionado problemas de identificação e classificação das espécies no gênero. Prova disso é que na literatura são encontrados mais de 600 nomes de espécies. Contudo, atualmente, são reconhecidas pouco mais de 250 espécies (Svenson 1939, González-Elizondo e Peterson 1997 e Govaerts *et al.* 2007). Torrey (1836) organizou o gênero *Eleocharis* em 7 subgêneros, considerando as espécies da América do Norte. Koyama (1961) moveu *E. margaritaceae* e *E. parvula* do gênero *Scirpus* para *Eleocharis* e criou a seção *Pauciflorae* em *Eleocharis*. Kunth (1937), baseado no trabalho de Nees (1834, 1835), dividiu o gênero em três grupos sem hierarquia definida: *Eleocharis Eleocharis* (incluindo *Eleocharis*, *Chaetocyperus* Nees e *Scirpidium* Nees), *E. Eleogenus* e *E. Limnocharis* Kunth.

Com o passar do tempo, várias espécies de *Eleocharis* foram descobertas e novas classificações deste gênero foram elaboradas e modificadas. Uma das mais completas foi feita por Svenson (1929, 1934, 1937 e 1939), que organizou o gênero em nove séries. Kukkonen (1990) propôs a organização de *Eleocharis* nos subgêneros: *Eleogenus* (Nees) C.B. Clarke, *Zinserlingia* T.V. Egorova, *Scirpidium* (Nees) Kukkonen, *Limnochloa* (P. Beauv. ex T. Lestib.) Torr. e *Eleocharis* R.Br. A revisão mais recente e mais aceita foi feita por González-Elizondo e Peterson (1997). Estes autores dividiram o gênero nos subgêneros *Scirpidium*, *Limnochloa*, *Zinserlingia* e *Eleocharis*, seis seções, oito séries e sete subséries (Tabela 1).

**Tabela 1.** Comparação entre as classificações para o gênero *Eleocharis*. Fonte: Yano *et al.* (2004)

Tanto a classificação de Svenson (1939), quanto as demais classificações como as de Koyama (1961), Kukkonen (1990) e González-Elizondo e Peterson (1997), levaram em consideração apenas caracteres ligados a macromorfologia, como forma dos aquênios e relação da largura entre a espiguetta e o colmo. Porém, trabalhos que fizeram a comparação destas classificações com dados moleculares, obtida pelo sequenciamento do “*Internal Transcribed Spacer*” (ITS) do rDNA, revelaram que não há correspondência total entre as classificações e as relações filogenéticas do grupo (Roalson e Friar 2000 e Yano *et al.* 2004). Os primeiros autores analisaram 38 espécies coletadas na América do Norte e compararam com as classificações Svenson (1939), Kukkonen (1990) e González-Elizondo & Peterson (1997). Uma das maiores

contradições envolveu o gênero *Websteria* S.H. Wrigth, o qual foi tratado como independente por alguns autores (González-Elizondo e Peterson 1997). Porém, este gênero ficou inserido no mesmo clado de *Eleocharis*. Além disso, este estudo suportou a idéia da monofilia nos subgêneros *Limnochloa* e *Zinserlingia* (*sensu* González-Elizondo & Peterson 1997) e séries parafiléticas ou polifiléticas no subgênero *Eleocharis*.

O estudo de Yano *et al.* (2004) foi feito com 24 espécies japonesas de *Eleocharis*, comparando as classificações propostas por Koyama (1961), Svenson (1939) e González-Elizondo & Peterson (1997). Os dados obtidos indicaram que a espessura da espiguetta em relação à espessura do colmo é uma característica mais importante para a taxonomia do que as características encontradas no aquênio. As análises também confirmaram o que foi observado por Roalson e Friar (2000), já que os subgêneros *Limnochloa* e *Zinserlingia* (*sensu* González-Elizondo e Peterson 1997) apareceram como monofiléticos. Além disso, muitas subdivisões do subgênero *Eleocharis* apareceram como parafiléticas ou polifiléticas, como por exemplo, a série *Tenuissimae*.

Em um estudo mais recente, as análises moleculares do nrDNA ITS e cpDNA das sequências *trnC-ycf6* e *ycf6-psbM* foram realizadas em 259 amostras de 154 espécies coletadas em todo mundo (Roalson *et al. in press*). Estes dados foram comparados com a classificação mais aceita (González-Elizondo & Peterson 1997). Dentre os quatro subgêneros, os principais contrastes entre as análises filogenéticas e a classificação taxonômica apareceram no subgênero *Eleocharis*, principalmente nas séries *Tenuissimae* e *Eleocharis*. Para os subgêneros *Scirpidium*, *Zinserlingia* e *Limnochloa*, com exceção de algumas espécies como *E. dulcis* (subg. *Limnochloa*) e *E.*

*acicularis* e *E. bonariensis* (subg. *Scirpidium*) que foram paraliléticas, os três subgêneros como um todo foram monofiléticos.

#### **1.4. Características citológicas de Cyperaceae e *Eleocharis***

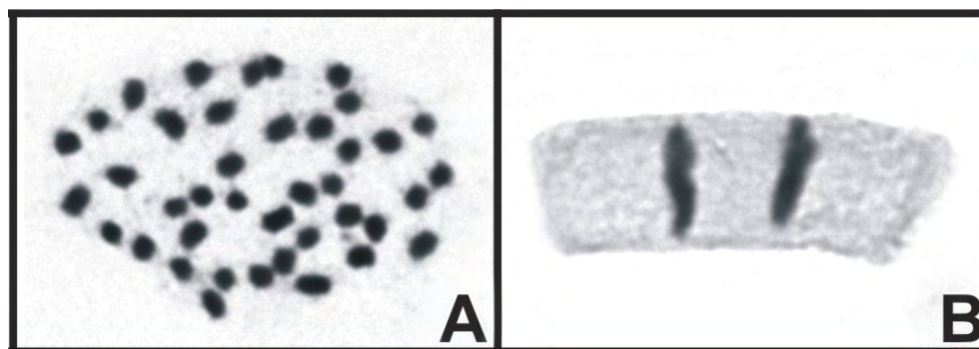
A família Cyperaceae possui três características citogenéticas conjuntas que são incomuns dentre os demais grupos de organismos: 1) cromossomos com centrômero difuso; 2) meiose pós-reducional e 3) formação de pseudomonades.

##### **1.4.1 Cromossomos holocêntricos**

Estes cromossomos não possuem constrição primária (Figura 7A) e o cinetócoro se organiza de modo difuso ao longo dos cromossomos, com exceção das regiões teloméricas, como demonstrado para *Luzula nivea* (Nagaki *et al.* 2005). Tais cromossomos foram descritos primeiramente em insetos (Scharader 1935), e somente em 1954 este tipo de cromossomo foi descrito no gênero *Eleocharis*, por Bataglia e Håkansson (veja Strandhede 1965). Além da família Cyperaceae, cromossomos holocêntricos foram descritos em representantes da família Juncaceae (Malheiros *et al.* 1947), apenas no gênero *Drosera* da família Droseraceae (Sheikh e Kondo 1995), em um subgênero de *Cuscuta* (Cuscutaceae) (Pazy e Plitmann 1994), em uma única espécie da família Liliaceae, *Chionographis japonica* Maxim. (Tanaka e Tanaka 1977) e em outra da família Myristicaceae, *Myristica fragrans* Houtt (Flach 1966). Além das plantas, cromossomos holocêntricos são encontrados em nematóides, como *Caenorhabditis elegans* Maupas (Buchwitz *et al.* 1999), em protozoários, algas e em várias ordens de insetos, incluindo Lepidoptera, Heteróptera e Odonata (Perez *et al.* 2000, Nokkala *et al.* 2002 e Wang e Porter 2004), e em espécies de alguns grupos de

aracnídeos, como, Acari, Araneae e Scorpiones (Araujo *et al.* 2008 e Schneider *et al.* 2009).

Na literatura, são encontradas diferentes denominações para este tipo de cromossomo. Autores como Håkansson (1958), Hoshino (1987) e Vanzela *et al.* (1996) o denominam cromossomo holocêntrico, no entanto, há autores que denominam este cromossomo como policêntrico (Bernardini 1959). Apesar do nome holocêntrico ser erroneamente empregado, esta denominação é amplamente aceita e utilizada. O termo mais correto seria holocinético, pois não existe centrômero distribuído ao longo do cromossomo, mas sim, atividade cinética. Esta característica leva à migração paralela dos cromossomos na anáfase mitótica (Figura 7B), como já demonstrado por Harms (1968) e Guerra *et al.* (2006). Em alguns casos, são observadas constrições em alguns cromossomos. Estas são chamadas constrições nucleolares, que são relativas às constrições secundárias dos cromossomos monocêntricos (Westerman e Colle 1984, Vanzela *et al.* 2000 e Da Silva *et al.* 2008a).



**Figura 7.** Cromossomos em coloração convencional com Giemsa A) Metáfase de *Eleocharis montana* ( $2n = 40$ ), uma espécie com cromossomos holocêntricos. Note que neste tipo de cromossomo não é possível observar constrições. B) Migração paralela dos cromossomos holocêntricos durante a anáfase da mitose em *Eleocharis sellowiana* ( $2n = 20$ ).

Cromossomos com cinetócoro difuso, quando natural ou artificialmente fragmentados e/ou fundidos, segregam normalmente na divisão celular, tanto na meiose quanto na mitose. Isto foi demonstrado por Castro *et al.* (1949), em *Luzula purpurea* (Juncaceae), por Håkansson (1954), em *Eleocharis palustris* (Cyperaceae) e em



*Rhynchospora pubera*, por Vanzela e Colaço (2002). Esses processos de quebra e fusão cromossômica em holocêntricos recebem a denominação de agmatoploidia (quebra) e simploidia (fusão), respectivamente (Luceño e Guerra 1997). Muito embora alguns trabalhos cite agmatoploidia tanto para quebra como para fusão cromossômica, em holocêntricos (Roalson 2008).

A permanência de cromossomos originados por fusão e fissão contribui para a extensa variação inter e intra-específica no número cromossômico em Cyperaceae, mesmo que poucas espécies tiveram seus números cromossômicos descritos. De cerca de 5.500 espécies de Cyperaceae (Govaerts *et al.* 2007), apenas 16% têm o número cromossômico conhecido, e metade destes números foram descritos para espécies de *Carex* L. (Roalson 2008). Apesar da escassez de conhecimento da evolução cromossômica nesta família (Hipp *et al.* 2009), a literatura indica que o processo de diferenciação dos cariótipos varia entre os grupos. Em *Carex*, agmatoploidia (quebra) e simploidia (fusão) são consideravelmente mais frequentes (Hipp *et al.* 2009), quando comparado com outros gêneros, como *Rhynchospora* Vahl (Luceño *et al.* 1998 e Vanzela *et al.* 2000), *Scleria* PJ Bergius (Yano e Hoshino 2007) e *Eleocharis* (Yano *et al.* 2004, Da Silva *et al.* 2008a e Da Silva *et al.* 2010), nas quais a poliploidia é a principal causa de alterações nos números cromossômicos.

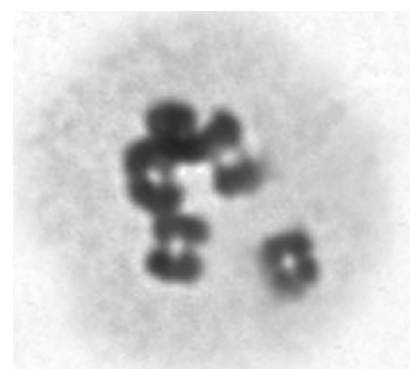
Neste contexto, não podemos descartar a possibilidade da existência de algum controle genético que favoreça a agmatoploidia e/ou simploidia em espécies diferentes. No entanto, a falta de estudos envolvendo um maior número de populações em uma mesma espécie, contribui para esta lacuna no conhecimento da evolução cromossômica em Cyperaceae. Estudos citogenéticos em diversas populações de uma determinada espécie podem revelar alterações cromossômicas que não são detectadas em amostras pequenas. As quais podem ser importantes na compreensão da evolução do cariótipo

desses grupos (Luceño e Castroviejo 1991, Hoshino e Okamura 1994, Ohkawa *et al.* 2000, Bureš *et al.* 2004 e Da Silva *et al.* 2008b).

Estudos citogenéticos em *Eleocharis* datam de antes de 1924, quando Piech descreveu o número de cromossomos de *E. prostes* (veja Nijalingappa 1973). Atualmente, a literatura tem registrado uma considerável variação no número cromossômico de espécies deste gênero, sendo de  $2n = 6$  para *E. subarticulata* (Da Silva *et al.* 2005) a  $2n = 200$  para *E. dulcis* (veja Roalson 2008). Variações intraespecíficas têm sido apontadas em algumas populações de *E. geniculata*, com  $2n = 10$  e  $20$  (Sanyal e Sharma 1972 e Nijalingappa 1973), *E. uniglumis* com  $2n = 46, 78-82$  e *E. palustris* com  $2n = 14-17, 38$  e  $39$ , (Bureš 1998 e Bureš *et al.* 2004), *E. aciculares* f. *longiseta* com  $2n = 20$  e  $21$  (Yano *et al.* 2004) e *E. maculosa* com  $2n = 10, 8, 7$  e  $6$  (Da Silva *et al.* 2008b). Apesar dessa variação, a maioria das espécies possui números múltiplos de 5 (Da Silva *et al.* 2008a, Roalson 2008) tal número,  $x = 5$ , foi proposto como o número básico para a família por Löve *et al.* (1957).

#### 1.4.2. Meiose nas Cyperaceae

A família Cyperaceae é conhecida pela ocorrência de meiose pós-reducional ou invertida. Este comportamento meiótico foi sugerido pela primeira vez em *Carex* por Heilborn (1928) e Wahl (1940). Este tipo de meiose, ocorre quando as cromátides irmãs apresentam orientação paralela na placa equatorial na metáfase I, migrando uma para cada lado na anáfase I. Com isso, o número de cromossomos não se reduz durante a primeira fase da meiose, já que o que se reduz é o número de



**Figura 8.** Cromossomos meióticos em coloração convencional com Giemsa em Metáfase I de *Eleocharis capillacea*, com cinco bivalentes formando estrutura em caixa.

cromátides de cada cromossomo. Nos cromossomos monocêntricos, as cromátides irmãs apresentarem orientação perpendicular ao eixo longo da placa equatorial antes da anáfase I, o que proporciona a separação dos cromossomos homólogos (meiose reducional).

O alinhamento dos bivalentes na meiose de holocêntricos gera uma configuração conhecida como “estrutura em caixa” (Figura 8) (Vanzela *et al.* 2000). Contudo, apenas a ocorrência desta estrutura não é prova de que a meiose é invertida. A análise de toda a meiose seria necessária para confirmar este comportamento. A meiose invertida foi bem documentada em *E. subarticulata*, na qual apesar da inexistência de bivalentes, pôde-se ver claramente que durante a primeira fase da meiose ocorre a separação das cromátides irmãs e, com isso, o número de cromossomos não é reduzido durante a anáfase I, ocorrendo a redução somente durante a segunda fase da meiose (Da Silva *et al.* 2005). Este tipo de comportamento meiótico foi sugerido para vários outros gêneros de Cyperaceae, como *Carex* (Wahl 1940), *Rhynchospora* (Vanzela *et al.* 2000) e também nos gêneros *Cuscuta*, da família Cuscutaceae (Pazi e Plitman 1994) e *Luzula*, da família Juncaceae (Nordenskiöld 1951). Além dos vegetais, alguns grupos de insetos que possuem cromossomos holocêntricos também apresentam meiose invertida; no entanto, diferentemente dos vegetais, nos quais este tipo de meiose ocorre para todos os cromossomos, em alguns grupos de insetos apenas os cromossomo sexuais, no sexo heterogamético, sofrem meiose invertida (Solari 1979).

Além de possuir cromossomos holocêntricos e meiose invertida, a família Cyperaceae exhibe também a ausência de tétrades com formação de pseudomônades, vista também na família Epacridaceae (Brown e Lemmon 2000). Ao final da meiose tradicional, os núcleos se organizam em uma tétrade (McCormick 1993) e são gerados ao final quatro grãos de pólen (gametófitos). Em Cyperaceae, no entanto, três dos quatro

núcleos são degenerados e apenas um torna-se funcional (Furness e Rudall 1999, San Martins *et al.* 2009). Após o amadurecimento da pseudomônade, apenas um androgametófito é formado (Furness e Rudall 1999).

### 1.5 Localização de DNA repetitivo

Existe uma grande variação tanto na quantidade quanto na disposição de bandas heterocromáticas em cromossomos de angiospermas, e esta variação pode ser vista até mesmo entre espécies próximas (Rego *et al.* 2009). Apesar das técnicas de bandamento mostrarem muita eficácia para a compreensão da organização cromossômica, tais estudos ainda são escassos em espécies vegetais com cromossomos holocêntricos.

Dentre as técnicas mais empregadas, o bandamento-C tem sido usado para localizar blocos de heterocromatina, enquanto que as colorações com fluorocromos DAPI (4'-6 diamidino - 2 fenilindol) e CMA<sub>3</sub> (cromomicina A<sub>3</sub>), são úteis para determinar a composição de bases de heterocromatina. O DAPI liga-se preferencialmente em regiões cromossômicas ricas em adenina e timina (A-T), enquanto CMA liga-se preferencialmente em regiões ricas em citosina e guanina (C-G). O uso sequencial destes fluorocromos permite entender a composição e a dinâmica de muitas famílias de DNA altamente repetitivas nos genomas (Fregonezi *et al.* 2006).

Alguns estudos têm demonstrado que cromossomos holocêntricos podem exibir blocos definidos de heterocromatina em regiões terminais e intersticiais (Westerman e Collet 1984; Panzera *et al.* 1992; Sheikh e Kondo 1995). De fato, o padrão de distribuição de heterocromatina em cromossomos holocêntricos parece ser similar ao encontrado em monocêntricos (Guerra 2000). Vanzela e Guerra (2000) descreveram para seis espécies de *Rhynchospora* (Cyperaceae) três tipos de heterocromatina, CMA<sup>+</sup>/DAPI<sup>-</sup>, CMA<sup>+</sup>/DAPI<sup>+</sup> e CMA<sup>-</sup>/DAPI<sup>+</sup>, que variaram em tamanho e

posicionamento nos cromossomos das espécies analisadas. No entanto, para sete espécies de *Eleocharis* foi observado apenas blocos de heterocromatina CMA<sup>-</sup>/DAPI<sup>+</sup> terminais (Da Silva *et al.* 2008a) nos cromossomos metafásicos mitóticos. Porém, quando estes cromossomos foram analisados em prófase, estas regiões de heterocromatina apresentaram-se dispersas ao longo dos cromossomos em pequenas porções.

Muitos segmentos repetidos de DNA têm sido precisamente detectados e mapeados fisicamente pela técnica de hibridação *in situ*. No entanto, os sítios de DNA ribossomal são, sem dúvida, os mais estudados por FISH em espécies vegetais e animais. Poucos estudos foram realizados em espécies vegetais com cromossomos holocêntricos. Em *Cuscuta approximata* (Cuscutaceae), sítios de DNAr 45S foram detectados em um par e sítios de DNAr 5S em três pares (Guerra e García 2004). Para a família Cyperaceae, vários sítios de rDNA 45S foram detectados em 8 espécies de *Rhynchospora* (Vanzela *et al.* 1998). Neste trabalho os autores encontraram uma grande variação no número de sítios; espécies com  $2n = 10$  apresentaram de 4 a 8 sítios e, até 30 sítios em espécies com  $2n = 50$ . Apesar desta variação numérica, o posicionamento dos sítios foi sempre terminal.

Furuta e Hoshino (1999) descreveram, para *Eleocharis mamillata* var. *cyclocarpa* ( $2n = 16$ ), 12 sinais terminais com a sonda de DNAr 18S e dois sinais intersticiais com sonda de DNAr 5S. Para outras sete espécies de *Eleocharis*, a sonda de DNAr 45S revelou sempre marcações terminais. Da Silva *et al.* (2008a) encontraram 10 marcações em *E. flavescens* ( $2n = 10$ ), oito em *E. sellowiana* ( $2n = 20$ ) e *E. acutangula* ( $2n = 54$ ) e quatro em *E. maculosa* ( $2n = 10$ ), *E. geniculata* ( $2n = 20$ ) e *E. montana* ( $2n = 40$ ). Para *E. subarticulata*, dos seis cromossomos do complemento diplóide, quatro revelaram sítios de DNAr 45S (Da Silva *et al.* 2005). A posição terminal destes sítios

permaneceu inalterada, até mesmo quando ocorrem rearranjos cromossômicos, como detectados em *E. maculosa*, na qual a simplóidia foi responsável pela redução cromossômica de  $2n = 10$  para  $2n = 8, 7$  e  $6$ . Neste trabalho também foram detectados dois sítios de DNAr 5S em cada cariótipo, porém, estes sítios foram terminais e intersticiais dependendo do cariótipo analisado (Da Silva *et al.* 2008b).

Além de sondas de DNAr, outras sondas foram utilizadas em estudos com espécies de Cyperaceae. Em *Rhynchospora tenuis*, hibridações *in situ* com a sonda telomérica (TTTAGGG) de *Arabidopsis thaliana* revelaram sinais apenas nas extremidades dos cromossomos de ambas as raças cromossômicas ( $2n = 4$  e  $8$ ), e sinais intersticiais não foram encontrados (Vanzela *et al.* 2003). Sinais intersticiais também não foram detectados em *E. maculosa* que sofreram simplóidia (Da Silva *et al.* 2008b). Porém, estudo realizado por Da Silva *et al.* (2005) com *Eleocharis subarticulata*, que possui um cariótipo originado por translocações múltiplas com apenas  $2n = 6$ , foram encontrados sítios ectópicos, adicionais aos sinais terminais.

## 2. Justificativa

Cyperaceae é a terceira maior família de monocotiledôneas (Trevisan e Boldrini 2008). O gênero *Eleocharis* é o quarto maior em número de espécies no Brasil. Muitas destas são endêmicas de áreas alagadas e importantes para a formação de biomassa nesses ecossistemas, como *E. interstincta* (Dos Santos e Esteves, 2005). Pelo menos duas espécies *E. acutangula* e *E. sellowiana* possuem atividade moluscicidas (Ruiz *et al.* 2005), já outras são utilizadas como ornamentação (*E. minima*) e muitas são consideradas ervas daninhas, principalmente em plantações de arroz.

O gênero *Eleocharis* apresenta inúmeras discordâncias quanto ao posicionamento das espécies em subgêneros, seções e séries, e isto tem trazido indefinições sobre os aspectos evolutivos do grupo (Roalson *et al. in press*). A escassez de informações citogenéticas em *Eleocharis*, e nas Cyperaceae de um modo geral, tem dificultado a compreensão de como tais cariótipos se organizam e se modificam. Tendo em vista isso, torna-se evidente a necessidade de ampliar tais conhecimentos neste grupo de plantas com cromossomos pouco comuns, não só com o auxílio de citogenética, mas também da taxonomia, morfologia e genética molecular.

### 3. Objetivos

Este trabalho tem como objetivo analisar, por parâmetros citogenéticos, moleculares e morfológicos, as relações entre as espécies do gênero *Eleocharis*, coletadas em vários estados do Brasil. Com estas análises, pretende-se esclarecer problemas taxonômicos e elaborar um modelo de diferenciação genético/cariotípica para um grupo vegetal com cromossomos especiais (holocêntricos).

#### 3.1 Objetivos Específicos

- (a) Coletar e identificar diferentes espécies e populações do gênero *Eleocharis* do território brasileiro para registrar a diversidade deste grupo;
- (b) Determinar o número e a morfologia cromossômica de diferentes espécies pertencentes a diversas populações estudadas;
- (c) Avaliar os mecanismos de diferenciação e evolução cariotípica em várias espécies de *Eleocharis*, pela aplicação das técnicas de coloração (i) convencional, (ii) bandamento cromossômico e (iii) hibridação *in situ*;
- (d) Utilizar diferentes marcadores moleculares (RAPD) para diferenciar espécies e populações que tenham problemas taxonômicos evidentes;
- (e) Elaborar um modelo evolutivo para o gênero baseado em características morfológicas e genéticas.



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**Capítulo 1**

Karyotypic diversification and its contribution to the  
taxonomy of *Eleocharis* (Cyperaceae) from Brazil

## Karyotypic diversification and its contribution to the taxonomy of *Eleocharis* (Cyperaceae) from Brazil

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**Abstract.** A karyotype analysis of 147 populations of 25 Brazilian species of *Eleocharis* (Cyperaceae) was carried out, including representatives of the three subgenera that occur in the country: *Limnochloa*, *Scirpidium* and *Eleocharis*. The analyses showed chromosomes without centromeres, but with terminal nucleolar constrictions (satellites) in some chromosomes. The chromosome numbers varied from  $2n=6$  in *E. subarticulata* and *E. maculosa* to  $2n=60$  in *E. laeviglumis*, but the chromosome basic number  $x=5$  was confirmed. Species of the subgenera *Eleocharis* and *Scirpidium* possess fewer and larger chromosomes, while those in the subgenus *Limnochloa* have small and more numerous chromosomes. These features indicate that the karyotypes of the subgenera *Eleocharis* and *Scirpidium* are more closely related, in agreement with morphological and phylogenetical data. The representatives of the section *Eleocharis* exhibited the largest differences in chromosome number and size, probably due to chromosome fission and fusion. Polyploidy was the most common event in this group. Nevertheless, most of the studied species exhibited regular meiosis with only bivalent formation, even the polyploids, such as in *E. geniculata* and *E. sellowiana*. The cytogenetic information obtained showed quite variable karyotypes with chromosomes gradually decreasing in size, and predominance of polyploidy. These results are useful in the differentiation of the subgenera.

### Introduction

*Eleocharis* R.Br. (Cyperaceae) is a genus with a worldwide distribution, including more than 200 species with a remarkable richness in South America (González-Elizondo and Tena-Flores 2000). The relatively simple morphology associated with a great phenotypic variation make difficult the identification and delimitation of the species (Simpson 1988; González-Elizondo and Peterson 1997; Trevisan and Boldrini 2008). There are few morphological characters that have taxonomic value in *Eleocharis* and several of them may be over-weighted, for example the ornamentation of the achenes and the number of style branches, which shows a high degree of homoplasy (González-Elizondo and Peterson 1997).

The supraspecific classifications that have been proposed for *Eleocharis*, for example, Svenson (1929, 1939), Kukkonen (1990) and González-Elizondo and Peterson (1997), are based on morphological data, and can be considered preliminary due to the scarce diagnostic features. When morphology is compared with the current phylogenetic hypothesis, it seems to be clear

that many morphological characters are either pleisiomorphic or have multiple origins (Roalson and Hinchliff 2007). To understand the evolutionary process in this group of plants, reviews are necessary that combine different approaches, as morphological, anatomical, cytogenetical and molecular markers, as suggested by González-Elizondo and Peterson (1997) and Roalson and Friar (2000). Molecular and cytogenetical analyses were useful to understand the evolutionary profile of two Cyperaceae genera from Japan. *Fimbritylis* Vahl was considered monophyletic and composed by three karyotype groups (Yano and Hoshino 2006a) and *Schoenoplectus* (Rchb.) with two clades with predominance of polyploidy (Yano and Hoshino 2005).

*Eleocharis*, as well as the other representatives of Cyperaceae, possess holocentric chromosomes, inverted meiosis and absence of tetrads with pseudomonad formation (Wahl 1940; Davies 1956; Strandhede 1965; Faulkner 1972). This family exhibits a wide range of chromosome numbers (Roalson 2008), originated by fusion (symploidy), fission (agmatoploidy) and polyploidy



**Table 1. Karyotype features of Brazilian species of *Eleocharis* R.Br. Species are grouped according to the supraspecific classification of González-Elizondo and Peterson (1997)**

Vouchers were deposited at the ICN herbarium of the Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil

Species	2n	Total length (µm)	L/S (µm) <sup>C</sup>	Coordinates, localities (voucher number)
<b>Subgenus <i>Eleocharis</i></b>				
<b>Section <i>Eleogenus</i> (Nees)</b>				
Benth. and Hook. f. (1883)				
<i>E. maculosa</i> (Vahl) Roem. and Schult.	10	10.86	2.3–1.8	30°12'08'S 50°12'52'W, Balneário Pinhal, RS (158385)
	8	18.75*	4.4–2.0	19°13'20'S 43°33'32'W, Serra do Cipó, MG (158386)
	7	19.04*	4.3–1.8	19°32'52'S 43°44'29'W, Jaboticatubas, MG (158390)
	6	19.04*	4.4–1.8	28°09'20'S 48°51'78'W, Apiaí, SP (158387)
<i>E. capillacea</i> Kunth <sup>A</sup>	10	7.44	1.6–1.2	24°21'58'S 49°02'06'W, Palmitalzinho, SP (158389)
				22°15'54'S 47°55'30'W, Brotas, SP (152642)22°36'45'S 50°23'57'W, Assis, SP (26)24°11'47'S 49°07'09'W, Bom Sucesso do Itararé, SP (239)19°13'20'S 43°33'32'W, Serra do Cipó, MG (152652; 152661) 19°04'13'S 43°24'58'W, Conceição do Mato Dentro, MG (133)
<i>E. flavescens</i> (Poir.) Urb.	10	6.70	1.4–1.1	25°50'53'S 48°32'50'W, Caiobá, PR (156144)
<i>E. geniculata</i> (L.) Roem. and Schult.	10	10.50	2.4–1.7	09°05'39'S 35°16'23'W, Japaratinga, AL (8)25°50'53'S 48°32'50'W, Caiobá, PR (156140)24°53'60'S 50°18'80'W, Ponta Grossa, PR (2)08°23'50'S 35°05'42'W Ipojuca, PR (10)
	20	14.77	1.7–1.2	20°34'51'S 51°36'36'W, Vestia, MS (69)07°45'10'S 34°51'38'W, Itamaracá, PE (9)23°14'03'S 44°45'28'W, Paraty, RJ (74)
<i>E. sellowiana</i> Kunth	10	6.70	2.0–1.2	25°17'48'S 49°54'42'W, Tibagi, PR (152018)25°28'74'S 49°52'93'W, São Luis do Purunã, PR (152019) 22°29'42'S 50°17'06'W, Echaporã, SP (4)26°16'46'S 51°03'24'W, Porto União, SC (84)29°48'18'S 50°03'45'W, Xangri-lá, RS (225)
	20	7.44	2.1–1.4	21°45'13'S 48°59'55'W, Ibitinga, SP (151980 and 3) 22°29'42'S 50°17'06'W, Echaporã, SP (151994) 22°32'51'S 50°21'18'W Assis, SP (152006)26°51'31'S 50°49'54'W, Caçador, SC (152011)26°14'56'S 49°54'27'W, Mafra, SC (152022)26°42'24'S 51°35'11'W, Irani, SC (204)25°50'53'S 48°32'50'W, Caiobá, PR (156143)25°17'48'S 49°54'42'W, Tibagi, PR (152018)24°57'40'S 53°26'12'W, Cascavel, PR (391)46°29'51'S 21°49'42'W, Poços de Caldas, MG (152645)19°04'13'S 43°24'58'W, Conceição do Mato Dentro, MG (152656)20°38'42'S 46°21'24'W, Furnas, MG (152663)19°32'52'S 43°44'29'W, Jaboticatubas, MG (152665)19°13'20'S 43°33'32'W, Serra do Cipó, MG (152669)21°34'08'S 45°46'77'W, Paraguaçu, MG (200)19°42'49'S 51°08'31'W, Paranaíba, MS (152672)20°10'30'S 51°12'54'W, Aparecida do Taboado, MS (62)30°06'18'S 50°44'33'W, Viamão, RS (191)30°12'08'S 50°12'52'W, Balneário Pinhal, RS (215)
<i>E. debilis</i> Kunth <sup>A</sup>	30	28.22	2.2–1.5	25°39'59'S 51°40'05'W, Pinhão, PR (153091)25°21'55'S 48°52'29'W, Morretes, PR (156128 and 156129)
<b>Section <i>Eleocharis</i></b>				
<i>E. minima</i> Kunth	20	15.66 <sup>B</sup>	2.0–1.2	26°36'54'S 51°04'18'W, Calmon, PR (152033)
	30	22.91 <sup>B</sup>	1.7–1.3	20°07'16'S 44°32'25'W, Itaúna, MG (152658)
	34	45.83 <sup>B</sup>	3.8–2.1	19°42'09'S 50°35'33'W, Alexandrita, MG (152674) 19°04'13'S 43°24'58'W, Conceição do Mato Dentro, MG (152654)25°17'248'S 49°54'42'W, Caçador, SC (152010)29°54'18'S 50°06'15'W, Imbé, RS (218)
<i>E. contracta</i> Maury <sup>A</sup>	20	19.42	2.2–1.5	26°51'31'S 50°49'67'W, Caçador, MG (152014) 25°17'48'S 49°54'42'W, Tibagi, PR (152096)

Table 1. (continued)

Species	2n	Total length (µm)	L/S (µm) <sup>C</sup>	Coordinates, localities (voucher number)
<i>E. nana</i> Kunth <sup>A</sup>	20	18.06	2.8–1.2	25°38'37"S 51°40'01"W, Pinhão, PR (158383)
<i>E. loefgreniana</i> Boeck. <sup>A</sup>	20	16.84	2.0–1.4	19°13'20"S 43°33'32"W, Serra do Cipó, MG (152659)
<i>Eleocharis</i> sp. 1 <sup>A</sup>	40	34.98	2.1–1.3	20°05'57"S 51°09'33"W, Aparecida do Taboado, MS (151987)19°10'55"S 57°42'47"W, Pantanal, MS (153095)20°38'42"S 46°21'24"W, Furnas, MG (158459)
<i>E. niederleinii</i> Boeck. <sup>A</sup>	20	13.86	1.6–1.7	25°38'37"S 51°40'01"W, Pinhão, PR (158380)19°42'09"S 50°35'33"W, Alexandrita, MG (151975)20°10'30"S 51°12'54"W, Aparecida do Taboado, MS (151991) 27°07'12"S 48°36'77"W, Rio Lavadeiras, SC (1) 30°06'18"S 50°44'33"W, Viamão, RS (192)
	30	33.10	6.5–0.5	26°16'46"S 51°03'02"W, Porto União, SC (152027) 25°41'06"S 51°32'08"W, Turvo, PR (152032)
<i>E. viridans</i> Kük. ex Osten <sup>A</sup>	20	17.88	2.2–1.3	30°06'18"S 50°44'33"W, Viamão, RS (153094)
	40	35.21	3.8–1.1	25°17'24"S 49°54'42"W, Caçador, SC (92)23°14'03"S 44°45'28"W, Paraty, RJ (75)26°49'67"S 50°21'54"W, Lebon Regis, SC (94)
<i>E. filiculmis</i> Kunth <sup>A</sup>	30	25.77	2.1–1.3	20°34'51"S 51°36'36"W, Vestia, MS (151969)19°42'49"S 51°08'31"W, Paranaíba, MS (151999)20°10'30"S 51°12'54"W, Aparecida do Taboado, MS (151986 and 151990)19°04'13"S 43°24'58"W, Conceição do Mato Dentro, MG (152657)20°00'54"S 49°03'18"W, Itapagipe, MG (151974)19°43'39"S 50°13'39"W, Alexandrita, MG (151978)49°03'18"S 20°00'54"W, Frutal, MG (152670)20°07'16"S 44°32'25"W, Itaúna, MG (114)19°13'20"S 43°33'32"W, Serra do Cipó, MG (141)22°15'54"S 47°55'30"W, Brotas, SP (152001) 22°32'51"S 50°21'18"W, Assis, SP (152005)26°51'83"S 50°49'67"W, Caçador, SC (152012)24°57'40"S 53°26'12"W, Cascavel, PR (389)
<i>E. montana</i> (Kunth) Roem. and Schult.	40	41.19	2.9–1.3	23°13'73"S 51°13'71"W, Londrina, PR (151964) 25°36'47"S 49°49'55"W, Lapa, PR (152023)25°36'16"S 51°40'74"W, Pinhão, PR (152028)21°45'13"S 48°59'55"W, Ibtinga, SP (151979)22°32'51"S 50°21'18"W, Assis, SP (24 and 152002)22°46'39"S 50°22'21"W, Cândido Mota, SP (77)26°51'83"S 50°49'67"W, Caçador, SC (152015)26°49'67"S 50°21'54"W, Lebon Regis, SC (152021)26°16'46"S 51°03'24"W, Porto União, SC (152034)19°53'15"S 49°22'39"W, Itapagipe, MG (151973)21°49'42"S 46°29'51"W, Poços de Caldas, MG (152643) 20°38'42"S 46°21'24"W, Furnas, MG (152662) 20°07'16"S 44°32'25"W, Itaúna, MG (152664) 19°04'13"S 43°24'58"W, Conceição do Mato Dentro, MG (138)20°34'51"S 51°36'36"W, Vestia, MS (67) 30°12'08"S 50°12'52"W, Balneário Pinhal, RS (213)
<i>E. subarticulata</i> (Nees) Boeck.	6	19.10*	3.8–2.2	21°49'42"S 46°29'51"W, Poços de Caldas, MG (152647) 25°17'24"S 49°54'42"W, Tibagi, PR (152031) 24°32'16"S 49°57'11"W, Piraí do Sul, PR (156093) 25°35'17"S 51°33'34"W, Pinhão, PR (156125) 25°22'51"S 48°51'50"W, Morretes, PR (156130) 25°28'74"S 49°52'93"W, São Luis do Purunã, PR (102) 29°03'57"S 50°06'19"W, Cambará do Sul, RS (236) 28°45'05"S 50°22'09"W, Cambará do Sul, RS (230)
Subgenus <i>Scirpidium</i> (Nees) Kukkonen				
<i>Eleocharis</i> sp. 2 <sup>A</sup>	10	8.47	1.9–1.5	25°39'59"S 51°40'05"W, Pinhão, PR (193)
<i>E. bonariensis</i> Nees <sup>A</sup>	20	20.20	2.5–1.6	28°45'05"S 50°22'09"W, Cambará do Sul, RS (229) 30°06'18"S 50°44'33"W, Viamão, RS (189)

(continued on next page)

Table 1. (continued)

Species	2n	Total length (µm)	L/S (µm) <sup>C</sup>	Coordinates, localities (voucher number)
Subgenus <i>Limnochloa</i>				
(P. Beauv. ex. Lestib.) Torr.				
<i>E. interstincta</i> (Vahl) Roem and Schult. <sup>A</sup>	40	22.92	1.4–0.9	08°39'40'S 35°09'57'W, Rio Formoso, PE (11)
	52	23.58	1.1–0.7	25°48'28'S 48°33'11'W, Matinhos, PR (158384)
				25°42'38'S 48°34'34'W, Matinhos, PR (374)
				20°14'06'S 49°10'12'W, Fronteira, MG (35)
<i>E. obtusetrigona</i> (Lindl. and Nees) Steud. <sup>A</sup>	52	24.40	1.1–0.7	19°4'13'S 43°24'58'W, Conceição do Mato Dentro, MG (152655)
				19°32'52'S 43°44'29'W, Jaboticatubas, MG (119)
				23°28'05'S 48°39'18'W, Paranapanema, SP (284)
				25°36'16'S 51°40'74'W, Pinhão, PR (354)
<i>E. liesneri</i> S. González and Reznicek <sup>A</sup>	50	24.24	1.3–0.7	21°34'08'S 45°46'77'W, Paraguaçu, MG (152648)
<i>E. plicarhachis</i> (Griseb.) Svenson <sup>A</sup>	54	30.64	1.5–0.9	19°10'55'S 57°42'47'W, Pantanal, MS (180)
				20°10'30'S 51°12'54'W, Aparecida do Taboado, MS (63)
				09°57'76'S 67°52'98'W, Rio Branco, AC (158)
<i>E. acutangula</i> (Roxb.) Schult.	54	30.63	1.3–1.0	19°43'39'S 50°13'39'W, Iturama, MG (151977)
				21°34'08'S 45°46'77'W, Paraguaçu, MG (152649)
				19°32'52'S 43°44'29'W, Jaboticatubas, MG (152666)
				20°00'54'S 49°03'18'W, Itapagipe, MG (36 and 38)
				19°42'09'S 50°35'33'W, Alexandrita, MG (41)
				20°38'42'S 46°21'24'W, Furnas, MG (112)
				19°4'13'S 43°24'58'W, Conceição do Mato Dentro, MG (134)
				46°29'51'S 21°49'42'W, Poços de Caldas, MG (148)
				22°29'42'S 50°17'06'W, Echaporã, SP (13)
				22°28'28'S 50°20'06'W, Assis, SP (19, 20 and 23)
				22°15'54'S 47°55'30'W, Brotas, SP (156)
				22°46'39'S 50°22'21'W, Cândido Mota, SP (78)
				20°05'57'S 51°09'33'W, Aparecida do Taboado, MS, (55 and 64)
				24°57'40'S 53°26'12'W, Cascavel, PR (390)
				25°31'35'S 49°19'45'W, Pirai do Sul, PR (158378)
				09°57'76'S 67°52'98'W, Rio Branco, AC (160)
<i>Eleocharis</i> sp. 3 <sup>A</sup>	42	22.31	1.2–0.8	08°39'40'S 35°09'57'W, Rio Formoso, PE (12)
<i>E. laevigulmis</i> R. Trevis. and Boldrini <sup>A</sup>	60	31.67	1.2–0.8	29°54'18'S 50°06'15'W, Imbé, RS (219)

\*Total length diploid.

<sup>A</sup>chromosome numbers recorded for the first time.<sup>B</sup>the chromosomes numbers are reported for the first time.<sup>C</sup>larger chromosome/smaller chromosome. All samples were collected by CRM Da Silva and ALL Vanzela.

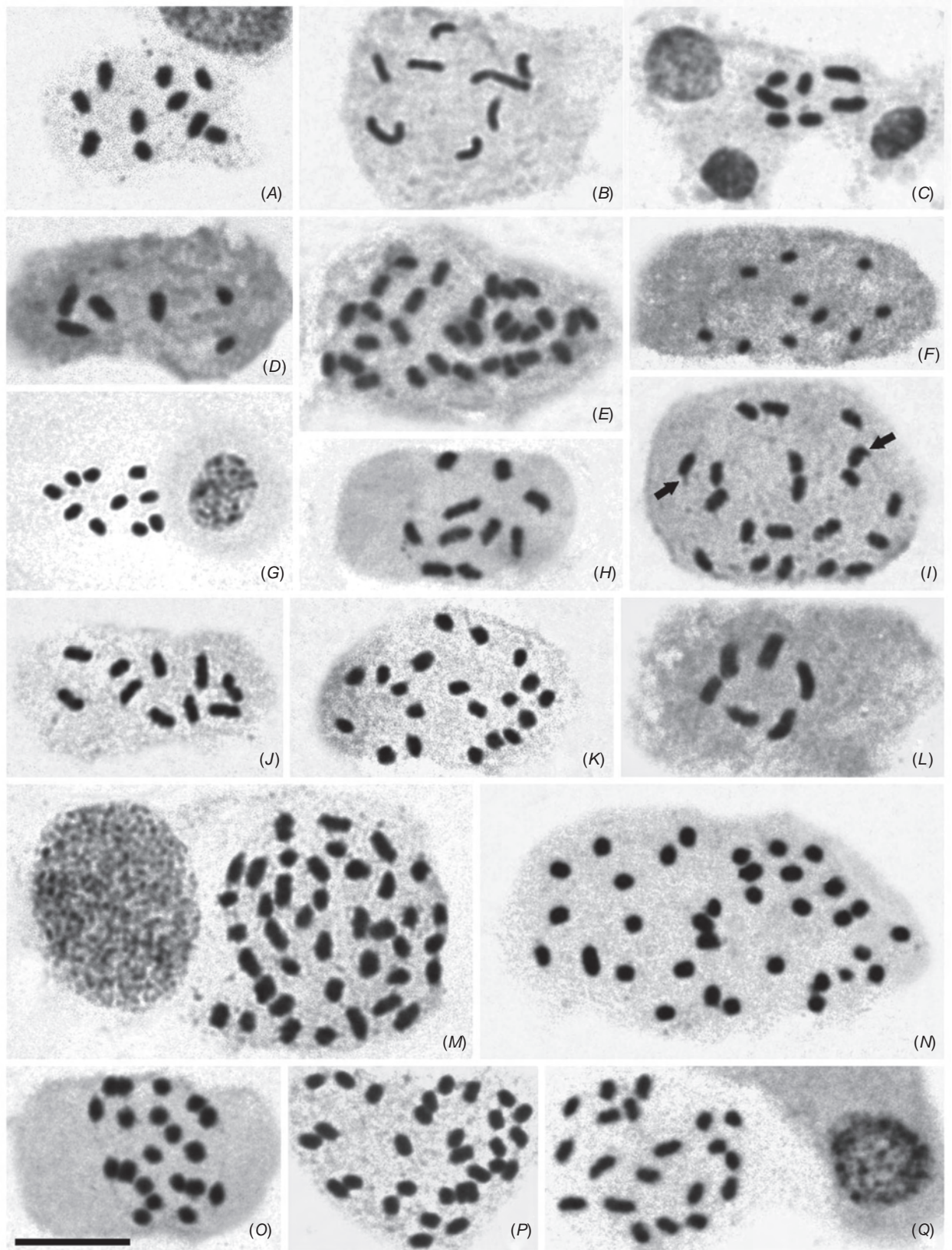
(Håkansson 1958; Strandhede 1965; Vanzela *et al.* 2000, 2003; Yano *et al.* 2004; Da Silva *et al.* 2008a; Hipp *et al.* 2009). In a smaller proportion, variation caused by multiple translocations and aneuploidy have been reported (Da Silva *et al.* 2005; Yano and Hoshino 2006b).

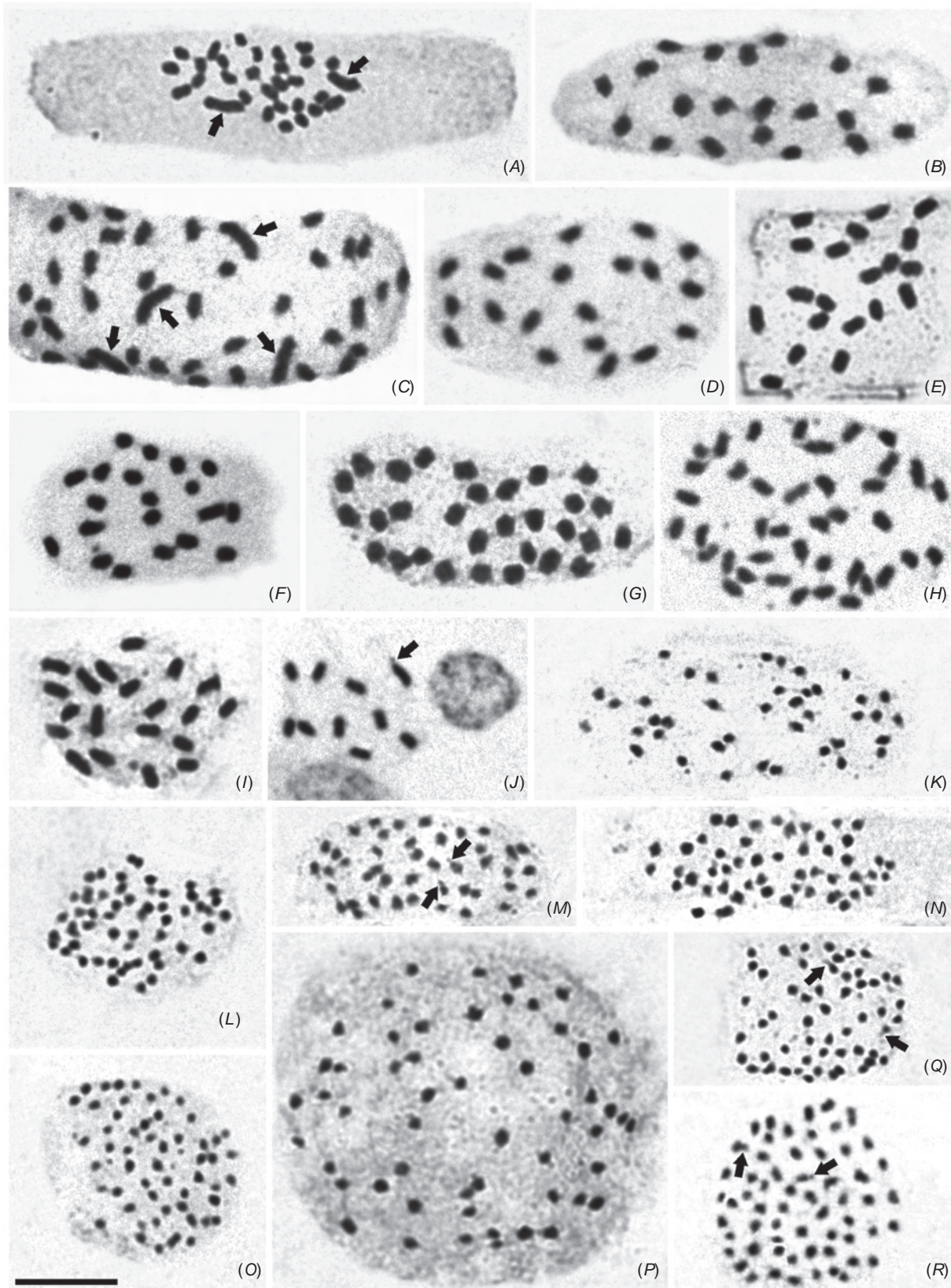
The more common mechanism of karyotype variation in *Eleocharis* appears to be polyploidy (Hoshino 1987; Yano *et al.* 2004; Da Silva *et al.* 2008a). The chromosome numbers described in the literature show a great variation, from  $2n=6$  (Da Silva *et al.* 2005) to  $2n=200$  (Roalson 2008). Further, some examples of intraspecific variation have been reported, such as  $2n=16, 38, 39, 46, 72-82$  in the *Eleocharis palustris* complex (Bureš *et al.* 2004). Yano *et al.* (2004) proposed a grouping of the *Eleocharis* species based in karyotype composition/organisation and molecular data. The first group was monophyletic and represented by karyotypes with small, symmetrical and

numerous chromosomes ( $<1.1\mu\text{m}$ ), which includes the subgenus *Limnochloa* (P. Beauv. ex T. Lestib.) Torr. The second group was paraphyletic and composed of species with larger chromosomes (1.4–4.3 µm) and a gradient of size or bimodal karyotypes, which includes species of subgenus *Eleocharis*.

At least 60 species of *Eleocharis* occur in Brazil, with representatives of three of the four subgenera: *Eleocharis*, *Scirpidium* (Nees) Kukkonen and *Limnochloa*, only *Zinserlingia* T. V. Egorova not being present in the country (González-Elizondo and Tena-Flores 2000; Alves *et al.* 2009; Trevisan and Boldrini 2008). *Eleocharis* is a genus with a special importance in the Brazilian flora because of a great number of species that grow in wetland areas, some of them widely distributed and others endemic. In this study we analysed the karyotypes of 25 species of *Eleocharis* by mitosis and meiosis

**Fig. 1.** Conventional chromosome analysis showing metaphases and prometaphases in species of the section *Eleogenus* (A–M): (A–D) *Eleocharis maculosa* with  $2n=10, 8, 7$  and  $6$ , respectively; (E) *E. debilis* with  $2n=30$ ; (F) *E. flavescens* with  $2n=10$ ; (G) *E. capillacea* with  $2n=10$ ; (H–I) *E. sellowiana* with  $2n=10$  and  $20$ , respectively. Arrows indicate the nucleolar constrictions; (J–K) *E. geniculata* with  $2n=10$  and  $20$ , respectively; (L) *E. subarticulata* with  $2n=6$ ; (M) *E. montana* with  $2n=40$ , and in the section *Eleocharis*. (N–Q): (N–P) *E. minima* with  $2n=34, 20$  and  $30$ , respectively; (Q) *E. niederleinii* with  $2n=20$ . Note the diffuse interphase nuclei without evident chromocenters in C, M and Q and with ones in G. Bar = 10 µm.





with the aim to compare the karyotype structure and evolution, as well as the usefulness of such information to the taxonomy of *Eleocharis*.

### Materials and methods

For this study 147 populations of 25 species, with at least three specimens of each population, were collected in 10 Brazilian states. Samples were cultivated in the greenhouse of the Laboratório de Biodiversidade e Restauração de Ecossistemas (LABRE) at Universidade Estadual de Londrina, Paraná, Brazil. Vouchers were deposited at the ICN herbarium of the Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil (Table 1).

The karyotypical analyses were performed with root tips pretreated in 2 mM 8-hydroxyquinoline for 24 h, fixed in ethanol:acetic acid (3:1, v:v) for 24 h, and stored at  $-20^{\circ}\text{C}$  or immediately used. Samples were washed in distilled water, softened in 4% cellulase plus 40% pectinase (w:v) at  $37^{\circ}\text{C}$  for 3 h, hydrolysed in 1 M HCl for 10 min at  $60^{\circ}\text{C}$ , washed again in distilled water, and squashed in a drop of 45% acetic acid. Slides were stained in 2% Giemsa and mounted with Entellan. The chromosome counts were made in at least 20 cells for each sample. The chromosome size and the haploid/diploid set length were measured from at least 10 metaphase cells with similar condensation, with the MicroMeasure 3.3 software ([www.colostate.edu/Depts/Biology/MicroMeasure](http://www.colostate.edu/Depts/Biology/MicroMeasure)) and the data used to assemble idiograms.

For the meiotic study, spikelets of at least one species of each section were dissected and the anthers directly fixed in absolute ethanol:acetic acid (3:1, v:v) for 12 h, and kept at  $-20^{\circ}\text{C}$  until used. Anthers were hydrolysed in 1 M HCl at  $60^{\circ}\text{C}$  for 9 min, washed in distilled water and squashed in a drop of 45% acetic acid. The coverslips were removed after freezing in liquid nitrogen, stained with 2% Giemsa and the slides mounted with Entellan. All images were acquired with a Leica DM 4500 B microscope equipped with a DFC 300FX camera and Leica IM50 4.0 software.

### Results and discussion

The chromosome number of 147 populations of 25 species, including representatives of all the subgenera of Brazilian *Eleocharis* (Table 1) was analysed. None of the species studied presented primary constrictions in the chromosomes and only terminal nucleolar constrictions (satellites) were visualised (Figs 1I, 2J, M, Q, R), as reported for other species of *Eleocharis*, as well as other Cyperaceae (Hoshino 1987; Luceño *et al.* 1998; Yano and Hoshino 2007; Da Silva *et al.* 2008a). For 17 species, the chromosome numbers are reported for the first time. These varied from  $2n=10$  in *E. capillacea* Kunth to  $2n=60$  in *E. laevigulumis* R. Trevis. and Boldrini. In the other eight species previously reported numbers were

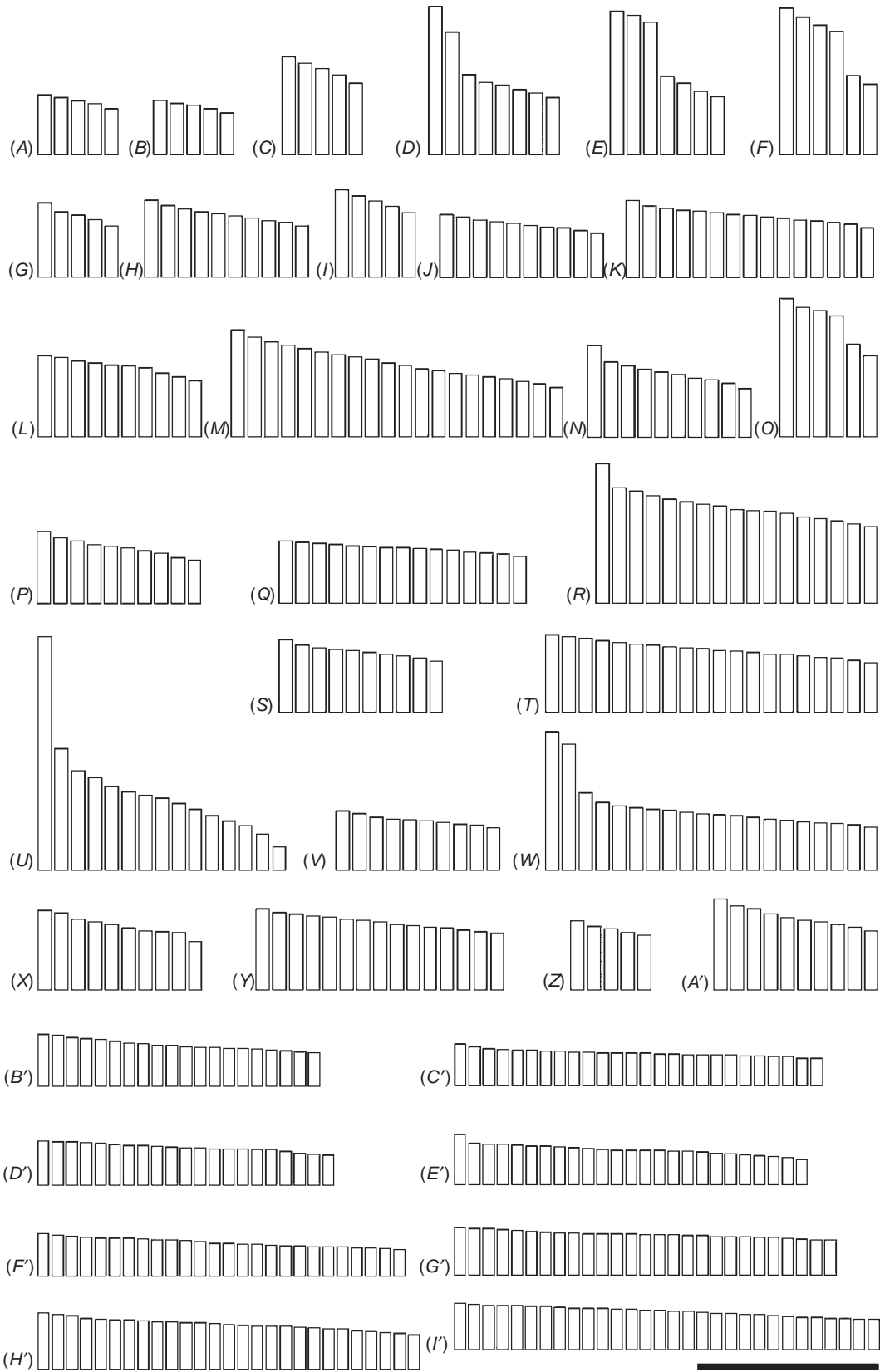
confirmed (Table 1). Of the more than 200 species of *Eleocharis* known worldwide, less than 25% have their karyotypes described (Davies 1956; Strandhede 1964, 1965; Harms 1968; Nijalingappa 1972, 1973; Sanyal and Sharma 1972; Schuyler 1977; Löve and Löve 1981; Hoshino 1987; Hoshino *et al.* 2000; Yano *et al.* 2004; Da Silva *et al.* 2005, 2008b; Yano and Hoshino 2006b; Roalson 2008). As for Brazil, of the 63 species listed at present (Alves *et al.* 2009), the chromosome numbers of 12 were previously described,  $2n=20$  in *E. geniculata* (Pedrosa *et al.* 1999) and six different numbers for 11 species:  $2n=6, 10, 12, 20, 40$  and  $54$  (Da Silva *et al.* 2008a).

Of ~5000 species in the family Cyperaceae (Goetghebeur 1998), only 16% have the chromosome number known, and half of these numbers have been described for species of *Carex* L. (Roalson 2008). Although there is a 'gap' in the knowledge of the patterns and processes of chromosomal evolution in this family (Hipp *et al.* 2009), the literature indicates that the karyotype differentiation process varies among groups. In *Carex*, agmatoploidy and symploidy are considerably more frequent (Hipp *et al.* 2009) when compared with other genera such as *Rhynchospora* Vahl (Luceño *et al.* 1998; Vanzela *et al.* 2000), *Scleria* P.J. Bergius (Yano and Hoshino 2007) and *Eleocharis* (Yano *et al.* 2004; Da Silva *et al.* 2008a). In this context, we cannot discard the possible existence of some genetic control that favours the agmatoploidy and/or symploidy in different species. However, the lack of studies involving a larger number of populations contributes to this gap in the knowledge of chromosomal evolution in Cyperaceae. Cytogenetic studies in several populations of one species, like those in *Carex laevigata* Smith (Luceño and Castroviejo 1991), *Carex conica* Boott (Hoshino and Waterway 1994), *Carex blepharicarpa* Franch. (Hoshino and Okamura 1994), *Carex sociata* Boott (Ohkawa *et al.* 2000), *Eleocharis palustris* (L.) Roem. and Schult. (Bureš *et al.* 2004) and *Eleocharis maculosa* (Vahl) Roem and Schult. Da Silva *et al.* (2008b) show that an increase in intraspecific sampling can reveal chromosome changes that are not detected in smaller samples, which can be important in understanding karyotype evolution of these groups.

### Subgenus *Eleocharis*

Of the 25 studied species, 16 belong to the subgenus *Eleocharis*, representing two of the three sections recognised by González-Elizondo and Peterson (1997): *Eleogenus* (Nees) Benth. and Hook. F. and *Eleocharis*. In section *Eleogenus*, six species were studied. The chromosome numbers varied from  $2n=6$  in *Eleocharis maculosa*, which also exhibited populations with  $2n=10, 8$  and  $7$  (Fig. 1A–D), as previously reported by Da Silva *et al.* (2008b), to  $2n=30$  in *Eleocharis debilis* Kunth (Fig. 1E). *Eleocharis flavescens* (Poir.) Urb. and *E. capillacea* Kunth

**Fig. 2.** Conventional chromosome analysis showing metaphases in species of the section *Eleocharis*. (A–H): (A) *E. niederleinii* with  $2n=30$ . Arrows point out two larger chromosomes; (B–C) *E. viridans* with  $2n=20$  and  $40$ . Arrows indicate four larger chromosomes. (D–F) *E. loefgreniana*, *E. contracta* and *E. nana*, all with  $2n=20$ . (G) *E. filiculmis* with  $2n=30$ , (H) *Eleocharis* sp. 1 with  $2n=40$ , and in the subgenus *Scirpidium*. (I–J): *E. bonariensis* with  $2n=20$  and *Eleocharis* sp. 2 with  $2n=10$ , respectively. Arrows point out nucleolar constrictions. Conventional chromosome analysis in species of subgenus *Limnochloa*. (K–R): (K–L) *Eleocharis interstincta* with  $2n=40$  and  $52$ , respectively; (M) *Eleocharis* sp. 3 with  $2n=42$ ; (N) *E. liesneri* with  $2n=50$ ; (O) *E. obtusetrigona* with  $2n=52$ ; (P) *E. plicarhachis* with  $2n=54$ ; (Q) *E. acutangula* with  $2n=54$  and (R) *E. laevigulumis* with  $2n=60$ . Arrows point out nucleolar constrictions. Bar = 10  $\mu\text{m}$ .



showed  $2n = 10$  (Fig. 1F,G). The other two species, *Eleocharis sellowiana* Kunth and *E. geniculata* (L.) Roem and Schult., also have intraspecific variation with two chromosome numbers, both with  $2n = 10$  and  $20$  (Fig. 1H–K).

In section *Eleocharis*, chromosome numbers varied from  $2n = 6$  in *E. subarticulata* (Nees) Boeck. (Fig. 1L) to  $2n = 40$  in *E. montana* (Kunth) Roem. and Schult. (Fig. 1M). *Eleocharis minima* (Vahl) Roem. and Schult. was the only species that showed three numbers with  $2n = 20, 30$  and  $34$  (Fig. 1N–P). Other species also presented multiple numbers, as *E. niederleinii* Boeck. with  $2n = 20$  and  $30$  (Figs 1Q, 2A) and *E. viridans* Kük. ex Osten with  $2n = 20$  and  $40$  (Fig. 2B,C). For the remaining species the numbers were  $2n = 20$  in *E. loefgreniana* Boeck., *E. contracta* Maury, and *E. nana* Kunth (Fig. 2D–F),  $2n = 30$  in *E. filiculmis* Kunth (Fig. 2G), and  $2n = 40$  (Fig. 2H) in one species related to *E. loefgreniana* but with very proliferous spikelets, named here as *Eleocharis* sp. 1. Proliferous spikelets, producing new culms instead of flowers, are not characteristic of *E. loefgreniana* and these samples could belong to an undescribed taxon.

The chromosome measurements revealed that all the species of subgenus *Eleocharis* have karyotypes with chromosomes decreasing gradually in size (Fig. 3). *Eleocharis niederleinii* with  $2n = 30$  (Fig. 3U) showed two chromosomes  $\sim 6.5 \mu\text{m}$  in length, and *E. viridans* with  $2n = 40$  (Fig. 3W) four chromosomes varying from  $3.8 \mu\text{m}$  to  $2.2 \mu\text{m}$  (Table 1, Fig. 3). These bigger chromosomes could have been originated by chromosome fusion or symploidy (which is a type of dispoloidy). This rearrangement seems to be common in other species of section *Eleocharis*, such as *E. palustris* (L.) Roem. and Schult., *E. uniglumis* (Link) Schult. and *E. mamillata* H. Lindb. (Strandhede 1965), but was better documented in *E. maculosa*, which is a member of the section *Eleogenus* (Da Silva *et al.* 2008b). However, our data do not allow us to identify, in this case, the chromosomes that participated in these fusions in the process of dispoloidy. In another case, Yano and Hoshino (2006b) reported the occurrence of aneuploidy in *Eleocharis kamtschatica* (C.A. Mey.) Kom. based in chromosome length differences, but these authors do not show the non-disjunction process or the irregular meiosis to confirm the aneuploidy process.

The representatives of the sections *Eleogenus* and *Eleocharis* from Brazil share common cytogenetic features including: (i) chromosome reduction to  $2n = 6$  starting from  $x = 5$ ; (ii) polyploidy in  $\sim 80\%$  of the species; (iii) occurrence of intraspecific variation in chromosome number; (iv) chromosome length around  $2.2 \mu\text{m}$ , except for *E. niederleinii* and *E. viridans*; and (v)  $\sim 20.42 \mu\text{m}$  of haploid total length. Similarly, other species of subgenus *Eleocharis* that occur in other continents show similar karyotype variation

as presented here (Håkansson 1958; Strandhede 1965; Nijalingappa 1973; Bureš *et al.* 2004; Yano *et al.* 2004; Roalson 2008). The diversity of karyotypes found in *Eleocharis* subgenus *Eleocharis* is mirrored by the tremendous morphological diversity in the group, many of whose subdivisions are para- or polyphyletic (Roalson and Friar 2000).

### Subgenus *Scirpidium*

Only two species of subgenus *Scirpidium* were analysed, *E. bonariensis* Nees with  $2n = 20$  (Fig. 2I) and *Eleocharis* sp. 2 with  $2n = 10$  (Fig. 2J), a species closely related to *E. radicans* (Poir.) Kunth, which was also recorded with  $2n = 10$  (Da Silva *et al.* 2008a). *Eleocharis* sp. 2 differs from *E. radicans* by having capillary culms, spikelets 15–30-flowered and smaller achenes, being probably a new taxon for the subgenus. Besides *E. radicans*, there are few cytogenetical data for species of *Scirpidium* in the literature, with records for only two additional species, *E. acicularis* (L.) Roem. and Schult. with  $2n = 56, 20$  and *E. atricha* R.Br. with  $2n = 10$  (Roalson 2008).

In spite of the low number of studied species, the intraspecific variation in *E. acicularis* and the chromosomes gradually decreasing in size that have been found, allow us to suggest that the karyotypes of *Scirpidium* and *Eleocharis* have greater similarity to each other than either is to karyotypes of subgenus *Limnochloa*. This conclusion corroborates the results of the phylogenetic studies of Roalson and Friar (2000) and Roalson and Hinchliff (2007). The morphological evidence also indicates that *Scirpidium*, although a well-delimited group with species sharing a particular ornamentation of the achenes (with longitudinal rows of fine trabeculae), is more closely related to the subgenus *Eleocharis* than to *Limnochloa*.

### Subgenus *Limnochloa*

All the species of subgenus *Limnochloa* exhibited karyotypes with many and small chromosomes. *Eleocharis interstincta* (Vahl) Roem. and Schult. presented  $2n = 40$  and  $52$ , being  $40$  the lowest found to this subgenus (Fig. 2K, L). Interestingly, we found  $2n = 42$  (Fig. 2M) in a species (named here as *Eleocharis* sp. 3), perhaps related to *E. interstincta* due to the presence of hollow and septate culms, but with an evident central vascular bundle along the culm. Since the septate culms have multiple origins in *Limnochloa* (Roalson and Hinchliff 2007) the relations between *Eleocharis* sp. 3 and *E. interstincta* need to be explored with other studies. The other numbers found in *Limnochloa* were  $2n = 50$  in *E. liesneri* S. González and Reznicek (Fig. 2N),  $2n = 52$  in *E. obtusetrigona* (Lindl. and Nees) Steud. (Fig. 2O),  $2n = 54$  in *E. plicarhachis* (Griseb.) Svenson and (Fig. 2P) and *E. acutangula* (Roxb.) Schult. (Fig. 2Q), and  $2n = 60$  in *E. laevigulmis* R. Trevis. and

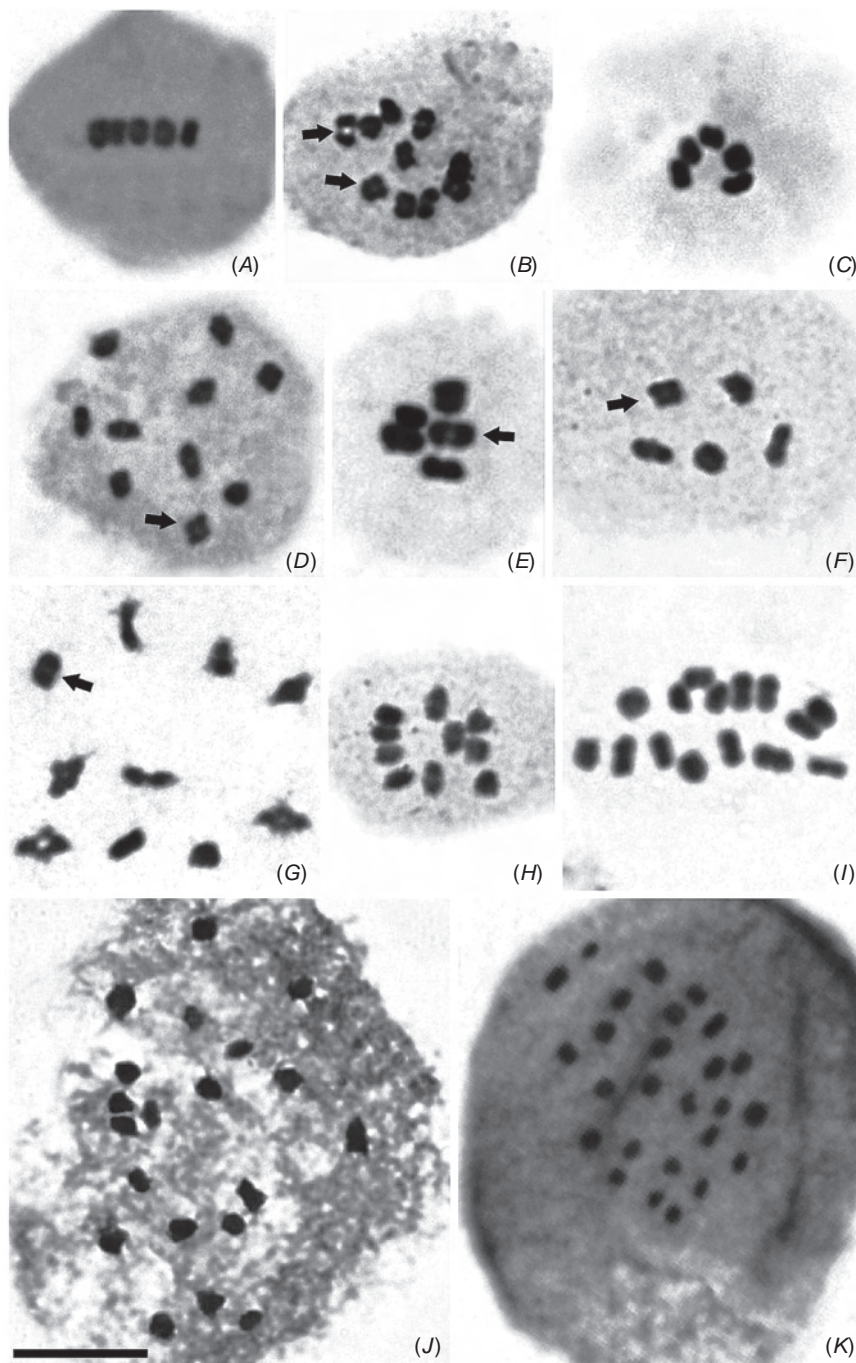
**Fig. 3.** Idiograms of the studied species. Note that all species exhibited chromosomes decreasing gradually in size, independent of chromosome numbers. All the idiograms represent the haploid set, except D–F and O, which indicate the diploid set. (A–K) Species of the section *Eleogenus*, being *E. flavescens* (A); *E. capillacea* (B); *E. maculosa* with  $2n = 10$  (C),  $2n = 8$  (D),  $2n = 7$  (E) and  $2n = 6$  (F); *E. sellowiana*  $2n = 10$  (G) and  $20$  (H); *E. geniculata* with  $2n = 10$  (I) and  $20$  (J); *E. debilis* (K). Species of the section *Eleocharis* (L–Y), being: *E. contracta* (L); *E. montana* (M); *E. nana* (N); *E. subarticulata* (O); *E. minima* with  $2n = 20$  (P),  $30$  (Q) and  $34$  (R); *E. loefgreniana* (S); *Eleocharis* sp. 1 (T); *E. niederleinii* with  $2n = 30$  (U) and  $20$  (V); *E. viridans* with  $2n = 40$  (W) and  $20$  (X), and *E. filiculmis* (Y). Species of the subgenus *Scirpidium* (Z, A'), being: *Eleocharis* sp. 2 (Z) and *E. bonariensis* (A'). Species of the subgenus *Limnochloa* (B'–I'), being: *E. interstincta* with  $2n = 40$  (B') and  $52$  (C'); *Eleocharis* sp. 3 (D'); *E. liesneri* (E'); *E. obtusetrigona* (F'); *E. acutangula* (G'); *E. plicarhachis* (H') and *E. laevigulmis* (I'). Bar =  $5 \mu\text{m}$ .



Boldrini (Fig. 2R). Of all these records, only *E. acutangula* had been previously studied showing always  $2n=54$  (Nijalingappa 1972; Rath and Patnaik 1978; Hoshino 1987; Da Silva *et al.* 2008a). The higher chromosome numbers found in *Eleocharis* worldwide belong to species of *Limnochloa*, as in *Eleocharis kuroguwai* Ohwi with  $2n \sim 196$

(Hoshino 1987), *E. ochrostachys* Steud. with  $2n=74$  (Yano *et al.* 2004), and *E. dulcis* (Burm.f.) Trin. ex Hensch. with  $2n=200$  and  $\sim 172$  (Roalson 2008).

Our cytogenetical analyses confirm that the species of *Limnochloa* are separated from the rest of the genus by their numerous, small chromosomes ( $<1.4 \mu\text{m}$ ), although the



**Fig. 4.** Meiotic analysis in species of *Eleocharis*: (A, B) metaphase I of *E. geniculata* with  $5^{\text{II}}$  and  $10^{\text{II}}$ , respectively; (C, D) metaphase I of *E. sellowiana* with  $5^{\text{II}}$  and  $10^{\text{II}}$ , respectively; (E, F) metaphase I of *E. flavescens* and *E. capillacea*, both with  $5^{\text{II}}$ ; (G) diakinesis in *E. nana* with  $10^{\text{II}}$ ; (H) metaphase I of *E. bonariensis* with  $10^{\text{II}}$ ; (I) metaphase I of *E. filiculmis* with  $15^{\text{II}}$ ; (J) metaphase I of *E. montana* with  $20^{\text{II}}$  and (K) metaphase I of *E. interstincta* with  $26^{\text{II}}$ . Arrows point out the 'Box' arrangement of bivalents. Bar =  $10 \mu\text{m}$ .

karyotypes do have chromosomes decreasing gradually in size as in the other species of the genus (see Table 1 and Fig. 3). The haploid set genome size of *Limnochloa* species seems not to exceed the sizes of those of other subgenera with lower chromosome numbers (Table 1). According to Yano *et al.* (2004), the karyotypes of *Limnochloa* can have been derived from others with  $2n = 10$  and  $20$ . Our results do not permit us to support or confirm the proposal of Yano and colleagues, but it seems clear that the karyotype differences among *Limnochloa* and the other subgenera corroborate the separation of *Limnochloa* as a group based on morphological features (Kukkonen 1990; González-Elizondo and Peterson 1997) and molecular data (Roalson and Friar 2000).

#### Meiotic analyses and polyploidy

The majority of the studied species presented chromosome numbers multiple of  $x = 5$ . *Eleocharis geniculata*, *E. sellowiana*, *E. flavescens* and *E. capillacea*, all belonging to section *Eleogenus* and all with  $2n = 10$ , show regular meiosis with five bivalents (Fig. 4A, C, E, F). The first two species presented also polyploid populations with  $2n = 20$ , but the regular meiosis with bivalent formation was also confirmed (Fig. 4B, D, respectively). In other species with chromosome numbers larger than  $2n = 10$ , such as *E. nana* with  $2n = 20$  (Fig. 4G), *E. bonariensis* with  $2n = 20$  (Fig. 4H), *E. filiculmis* with  $2n = 30$  (Fig. 4I), *E. montana* with  $2n = 40$  (Fig. 4J) and *E. interstincta* with  $2n = 52$  (Fig. 4K), only bivalents were observed. Additionally, some bivalents exhibited a 'box' shape, as in *E. geniculata* (Fig. 4B), *E. sellowiana* (Fig. 4D), *E. flavescens* (Fig. 4E), *E. capillacea* (Fig. 4F) and *E. nana* (Fig. 4G), which are characteristic of holocentric chromosomes (Strandhede 1965; Vanzela *et al.* 2000).

Our study showed 80% polyploidy in Brazilian *Eleocharis*, with bivalent associations in both diploids and polyploids. As the bivalent or multivalent associations may depend on the neopolyploid or paleopolyploid condition (Grant 1971; Otto 2007), as well as of gene control (Evans 1988; Griffiths *et al.* 2006; Bozza and Pawlowski 2008), our results do not allow us to determine if the chromosome numbers were doubled by auto- or allopolyploidy. Some multivalent associations have been associated with natural allopolyploids, as in the *E. palustris* complex, *E. uniglumis* and *E. mamillata* (Strandhede 1965) and in hybrids of *Carex* (Luceño 1993). Similarly, multivalent associations have been found in some autopolyploids, as in *Fimbristylis falcata* (Vahl) Kunth (Nijalingappa 1977), *Carex siderosticta* Hance (Tanaka 1940) and *Rhynchospora tenuis* Link (Vanzela *et al.* 1996). In the last case, normal bivalents were only rarely observed.

The cytogenetic information obtained here shows quite variable karyotypes with chromosomes gradually decreasing in size, independently of the chromosome numbers, and the predominance of polyploidy. These results are useful in the differentiation of the subgenera, but when the comparisons are done within a subgenus, more detailed cytogenetical analyses (chromosome banding and cyto-molecular tools) are required. Future studies should combine these tools with molecular data to better understand the evolution of *Eleocharis* and related genera.

#### Acknowledgements

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**Capítulo 2**

Cytogenetical and cytotaxonomical analysis of some  
Brazilian species of *Eleocharis* (Cyperaceae)

## Cytogenetical and cytotaxonomical analysis of some Brazilian species of *Eleocharis* (Cyperaceae)

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**Abstract.** Karyotype analysis of 21 samples of 11 species of *Eleocharis* (Cyperaceae) from 10 localities in Brazil, showed the presence of chromosomes without primary constrictions and parallel movement of chromatids at metaphase–anaphase transition. Only the terminal nucleolar constrictions (satellites) were visualised. The chromosome numbers varied from  $2n = 6$  in *E. subarticulata* to  $2n = 54$  in *E. acutangula*, but the chromosome basic number  $x = 5$  was confirmed. Generally, C-CMA<sub>3</sub><sup>+</sup> bands appear mostly in the extremities of the chromosomes, associated to NOR, and interstitial C-CMA<sub>3</sub> bands were found only in *E. geniculata* and *E. acutangula*. C-DAPI<sup>+</sup> bands were not found. Fluorescence *in situ* hybridisation (FISH) with the 45S rDNA probe was performed in five species. The results showed from four to eight hybridisation signals, always terminal. The analysed species include representatives of the following three subgenera of *Eleocharis* that occur in Brazil: *Limnochloa*, *Scirpidium* and *Eleocharis*. Species from the subgenus *Limnochloa* have small and numerous chromosomes. The remaining species, belonging to subgenera *Eleocharis* and *Scirpidium*, possess fewer and larger chromosomes. In subgenus *Eleocharis*, karyotypes of the section *Eleocharis* were differentiated by symploidy, agmatoploidy and polyploidy, whereas species of the section *Eleogenus* were all polyploids. Polyploidy seems to be the most frequent event in the karyotype differentiation in *Eleocharis*, but changes in the chromosome size and repetitive DNA sites were also observed.

### Introduction

*Eleocharis* comprises ~200 species, which are frequently found in flooded areas (Svenson 1929; González-Elizondo and Peterson 1997). *Eleocharis* is distinguished from the other genera of Cyperaceae by possessing stems with leaves reduced to a basal, tubular sheath, persistent style base, and inflorescence reduced to a simple terminal spikelet (González-Elizondo and Peterson 1997). In spite of the easy recognition of *Eleocharis* as a genus in the field and its prominent delimitation within Cyperaceae (Kukkonen 1990), the species are difficult to identify and classify because of the limited number of morphological features. The supraspecific classification of *Eleocharis* has been revised and modified in recent years (Faria 1998) and the more global classifications are those proposed by Kukkonen (1990) and González-Elizondo and Peterson (1997), on the basis of the widely accepted classification of Svenson (1929, 1932, 1934, 1937, 1939).

Most of the cytogenetical knowledge in Cyperaceae comes from studies in *Carex* and *Rhynchospora* (Davies 1956; Faulkner 1972; Luceño *et al.* 1998; Vanzela *et al.* 2000). These and other authors accept that the species of Cyperaceae possess uncommon cytogenetical features such as (i) holocentric chromosomes, (ii) post-reductional meiosis, (iii) absence of tetrads, owing to

degeneration of three of the four nuclei during pollen mitosis and (iv) karyotype evolution on the basis of agmatoploidy (fission), symploidy (fusion) and polyploidy. The occurrence of holocentric chromosomes in *Eleocharis* was proposed by Håkansson (1954), after treating samples of *E. palustris* with X-ray and the observation of the normal kinetic behaviour of the chromosome fragments produced in subsequent cellular cycles. Greilhuber (1995) suggested that the occurrence of holocentric chromosomes is a synapomorphy of the Cyperaceae. Recently, Guerra *et al.* (2005) studied the behaviour of holocentric chromosomes of *Rhynchospora tenuis* (Cyperaceae) by immunodetection with anti-tubulin, and found that during the metaphase, the microtubules are attached along the whole chromosome extension, typical of holokinetic chromosomes.

Cytogenetical studies in *Eleocharis* date from the early 1920s, when Piech described the chromosome number of *E. palustris* (Nijalingappa 1973). There is considerable variation in the chromosome numbers in the genus, from  $2n = 6$  (da Silva *et al.* 2005) to  $2n = \sim 196$  (Hoshino 1987); however, most of the species present the numbers  $2n = 10, 20, 30$  and 40, associated with the possible basic number  $x = 5$ . Intraspecific differences in the chromosome numbers were reported in *E. geniculata* with  $2n = 10$  and 20, in *E. atropurpurea*

with  $2n = 20$  and  $21$  (Nijalingappa 1973) and *E. palustris* with  $2n = 15$  and  $16$  (Strandhede 1964), which originated by agmatoploidy/symploidy. More recently, Bureš *et al.* (2004) showed that *E. palustris* subsp. *palustris* has  $2n = 16$ , rarely  $15$ , whereas *E. palustris* subsp. *vulgaris* frequently has  $2n = 38$  and can range from  $36$  to  $42$ . Yano *et al.* (2004) showed the occurrence of mixoploidy in the Japanese *E. acicularis* f. *longiseta* ( $2n = 20$  and  $21$ ). These authors, on the basis of cytogenetical and molecular studies, have proposed a cytological division of the genus into the following two groups: (i) species with karyotypes composed of small and numerous chromosomes (*E.* section *Limnochloa*) and (ii) species with big and less numerous chromosomes (other sections).

Some studies about repetitive DNA segments in members of the Cyperaceae report the existence of numerous GC-rich heterochromatic blocks in *Carex* (Greilhuber 1995) and the occurrence of different heterochromatin types (C-Giemsa<sup>+</sup>, CMA<sub>3</sub><sup>+</sup>/DAPI<sup>-</sup>, CMA<sub>3</sub><sup>+</sup>/DAPI<sup>+</sup> and CMA<sub>3</sub><sup>-</sup>/DAPI<sup>+</sup>) in *Rhynchospora* (Vanzela and Guerra 2000). FISH with 45S rDNA probes in members of Cyperaceae was performed on eight species of *Rhynchospora* by Vanzela *et al.* (1998). These authors found large variation in the number of sites, but stability in localisation, since all the sites were terminal. FISH with rDNA probes was also performed in *Eleocharis* and *Carex* by Furuta and Hoshino (1999), who found that in *E. mamillata* var.

*cyclocarpa* ( $2n = 16$ ) the 18S rDNA probe hybridised in 12 of the 16 chromosomes of the complement, always in the terminal positions. Terminal 45S rDNA sites were also found in *E. subarticulata* with  $2n = 6$  (da Silva *et al.* 2005). FISH with the 5S rDNA probe hybridised in the interstitial positions of two chromosomes. In *Carex pachygyna* and *C. ciliato-marginata*, both with  $2n = 12$ , the 5S rDNA probe localised segments in the terminal and interstitial regions and 18S rDNA probe always hybridised in the terminal regions. These examples indicate that these repetitive DNA families are dynamics and present a great potential to study the karyotype differentiation and evolution in this genus.

In the present study, the karyotypes of 21 samples of 11 species of *Eleocharis* (Cyperaceae) from 10 localities in Brazil were analysed. The aim of the study was to contribute to knowledge on the relationship between size, chromosome number and taxonomic grouping, such as those proposed by Yano *et al.* (2004). We also focused on the importance of DNA segment repeats in karyotype differentiation in *Eleocharis*.

### Materials and methods

Twenty-one samples representing 11 species of *Eleocharis* were collected in 10 different localities of south-eastern and southern Brazil. Vouchers were deposited in herbarium FUEL. In a few localities, more than one species was collected (Table 1).

**Table 1. Chromosome count and size in Brazilian species of *Eleocharis***  
Species are grouped according to González-Elizondo and Peterson (1997)

Species	$2n$	Size ( $\mu\text{m}$ )	Localities (coordinates) and voucher number
Subgenus <i>Eleocharis</i> S.González & P.M.Peterson			
Section <i>Eleocharis</i> S. González & P.M.Peterson			
<i>E. subarticulata</i> (Nees) Boeckl.	6	3.0–1.5	Ponta Grossa, Paraná, (25°05'06"S, 50°09'57"W), 35.411.
<i>E. minima</i> Kunth <sup>A</sup>	12	2.7–0.9	Sapopema, Paraná, (23°53'06"S, 50°35'24"W), 35.403.
<i>E. montana</i> (Kunth) Roem. & Schult <sup>A</sup>	20	2.3–1.3	Bela Vista do Paraíso, Paraná (23°01'36"S, 51°12'36"W), 36.079; Bela Vista do Paraíso, Paraná 36.081; Rolândia, Paraná (23°19'48"S, 51°22'21"W), 35.422; Assaí, Paraná (23°22'12"S, 50°49'48"W), 36.073; Sapopema, Paraná, 36.064.
<i>E. elegans</i> (HBK) R & S <sup>A</sup>	40	2.2–1.0	Assaí, Paraná, 36.074.
Section <i>Eleogenus</i> (Nees) Benth. & Hook.			
<i>E. flavescens</i> (Poir.) Urban <sup>A</sup>	10	2.0–1.3	Rolândia, Paraná, 35.424.
<i>E. maculosa</i> (Vahl) Roem & Schult. <sup>A</sup>	10	2.4–1.6	Florianópolis, Santa Catarina (27°34'12"S, 48°31'48"W), 35.406; Tibagi, Paraná (24°32'06"S, 50°24'18"W), 35.434.
<i>E. sellowiana</i> Kunth. <sup>A</sup>	20	2.6–1.4	Ibitinga, São Paulo (21°45'11"S, 49°01'33"W), 35.429; Rolândia, Paraná, 35.426; Bela Vista do Paraíso, Paraná, 36.080.
<i>E. geniculata</i> (L.) Roem & Schult.	20	3.2–4.1	Ponta Grossa, Paraná (24°59'24"S, 49°59'24"W), 36.084.
Subgenus <i>Limnochloa</i> (P.Beauv. ex Lestib.) Torr.			
Section <i>Limnochloa</i> (P.Beauv. ex T.Lestib.) Benth. & Hook. f.			
<i>E. brasiliensis</i> Boeckl <sup>A</sup>	~54 <sup>B</sup>		Biological collection of Unicamp, Campinas, São Paulo (22°48'48"S, 47°04'12"W), 35.405.
<i>E. acutangula</i> (Roxb.) Schult.	54	1.2–0.7	Rio Claro, São Paulo (22°24'54"S, 22°24'55"W), 35.455; Rolândia, Paraná, 35.420; Assaí, Paraná, 36.075; Sapopema, Paraná, 36.068.
Subgenus <i>Scirpidium</i> (Nees) Kukk.			
Section <i>Scirpidium</i> (Nees) Benth. & Hook. f.			
<i>E. radicans</i> (Poir.) Kunth. Enum. <sup>A</sup>	10		Sapopema, Paraná, 35.408.

<sup>A</sup>First chromosome counts.

<sup>B</sup>Approximate chromosome number.

Samples were cultivated in the nursery of the Laboratório de Biodiversidade e Restauração de Ecossistemas at Universidade Estadual de Londrina, Londrina, Brazil.

Karyotype analyses were performed with root tips pre-treated in 2 mM 8-hydroxyquinoline for 24 h, fixed in ethanol:acetic acid (3:1, v:v) for 24 h, and stored at  $-20^{\circ}\text{C}$  or immediately used. For the conventional staining, the root tips were washed in distilled water, softened in 4% cellulase plus 40% pectinase at  $37^{\circ}\text{C}$  for 3 h, hydrolysed in 1 M HCl for 10 min at  $60^{\circ}\text{C}$ , washed again in distilled water, and squashed in a drop of 45% acetic acid. Slides were stained in 2% giemsa and mounted with Entellan (Merck). The chromosome counts were made in at least 20 cells for each sample. For calculating the chromosome sizes, at least 10 cells with good chromosome morphology and similar condensation and spreading ability were measured, and the mean length was calculated for each pair. To test the parallel migration of the holocentric chromosomes in anaphases, slides were prepared with meristems obtained without a previous treatment with anti-mitotic agent and directly fixed in ethanol:acetic acid, as described above.

For chromosome banding, fixed samples were washed in distilled water, softened in 4% cellulase plus 40% pectinase at  $37^{\circ}\text{C}$  for 3 h, washed again and squashed in a drop of 45% acetic acid, without acid hydrolysis. After freezing in liquid nitrogen for coverslip removal, preparations were aged for later C-CMA<sub>3</sub> banding or immediately used for FISH with the 45S rDNA probe. For C-CMA<sub>3</sub> banding, preparations were treated according to Schwarzacher *et al.* (1980), with modifications. Slides were incubated in 45% acetic acid at  $60^{\circ}\text{C}$  for 10 min, 5% Ba(OH)<sub>2</sub> at  $25^{\circ}\text{C}$  for 10 min and  $2 \times \text{SSC}$  pH 7.0 at  $60^{\circ}\text{C}$  for 1 h and 30 min, and to finish, the samples were air-dried. After 3 days, samples were stained with a drop of  $0.5 \mu\text{g} \mu\text{L}^{-1}$  CMA<sub>3</sub> containing 2.5 mM MgCl<sub>2</sub> for 1 h 30 min, washed in distilled water and subsequently stained with  $2 \mu\text{g} \mu\text{L}^{-1}$  DAPI for 30 min. Slides were mounted in a solution of glycerol:McIlvaine buffer, pH 7.0, (1:1, v:v) containing 2.5 mM MgCl<sub>2</sub>.

FISH was carried out with the pTa71 probe containing 45S rDNA isolated from wheat (Gerlach and Bedbrook 1979). The probe was labelled with biotin-14-dATP by nick translation. Material was incubated in RNase ( $100 \mu\text{g} \mu\text{L}^{-1}$ ), post-fixed in 4% (w/v) paraformaldehyde, dehydrated in a 70% and 100% graded ethanol series for 5 min each and air-dried. A hybridisation mix ( $30 \mu\text{L}$  per slide), containing 100 ng of labelled probe ( $4 \mu\text{L}$ ), 100% formamide ( $15 \mu\text{L}$ ), 50% polyethylene glycol ( $6 \mu\text{L}$ ), 10% SDS ( $1 \mu\text{L}$ ), 100 ng of calf thymus DNA ( $1 \mu\text{L}$ ) and  $20 \times \text{SSC}$  ( $3 \mu\text{L}$ ), was denatured at  $70^{\circ}\text{C}$  for 10 min and immediately chilled on ice. Chromosome denaturation/hybridisation was performed at  $90^{\circ}\text{C}$  for 10 min,  $50^{\circ}\text{C}$  for 10 min and  $38^{\circ}\text{C}$  for 5 min with a thermal cycler,

and kept at  $37^{\circ}\text{C}$  overnight in a moist chamber. Post-hybridisation washes were performed at 80% stringency in  $2 \times \text{SSC}$ ,  $0.1 \times \text{SSC}/20\%$  formamide,  $0.1 \times \text{SSC}$ ,  $2 \times \text{SSC}$  and  $4 \times \text{SSC}/0.2\%$  Tween 20, at  $42^{\circ}\text{C}$  for 5 min each. The biotin-labelled probe was detected with avidin FITC-conjugate, and slides were mounted in an antifade solution (2.3% of 1,4-diaza-bicyclo(2.2.2)-octane, 20 mM TRIS-HCl, pH 8.0, and 90% glycerol, in distilled water) containing propidium iodide ( $1 \mu\text{g} \mu\text{L}^{-1}$ ). Images were taken with a Leica DM 4500 B microscope equipped with a DFC 300FX camera and Leica IM50 4.0 software (all from Leica Microsystems).

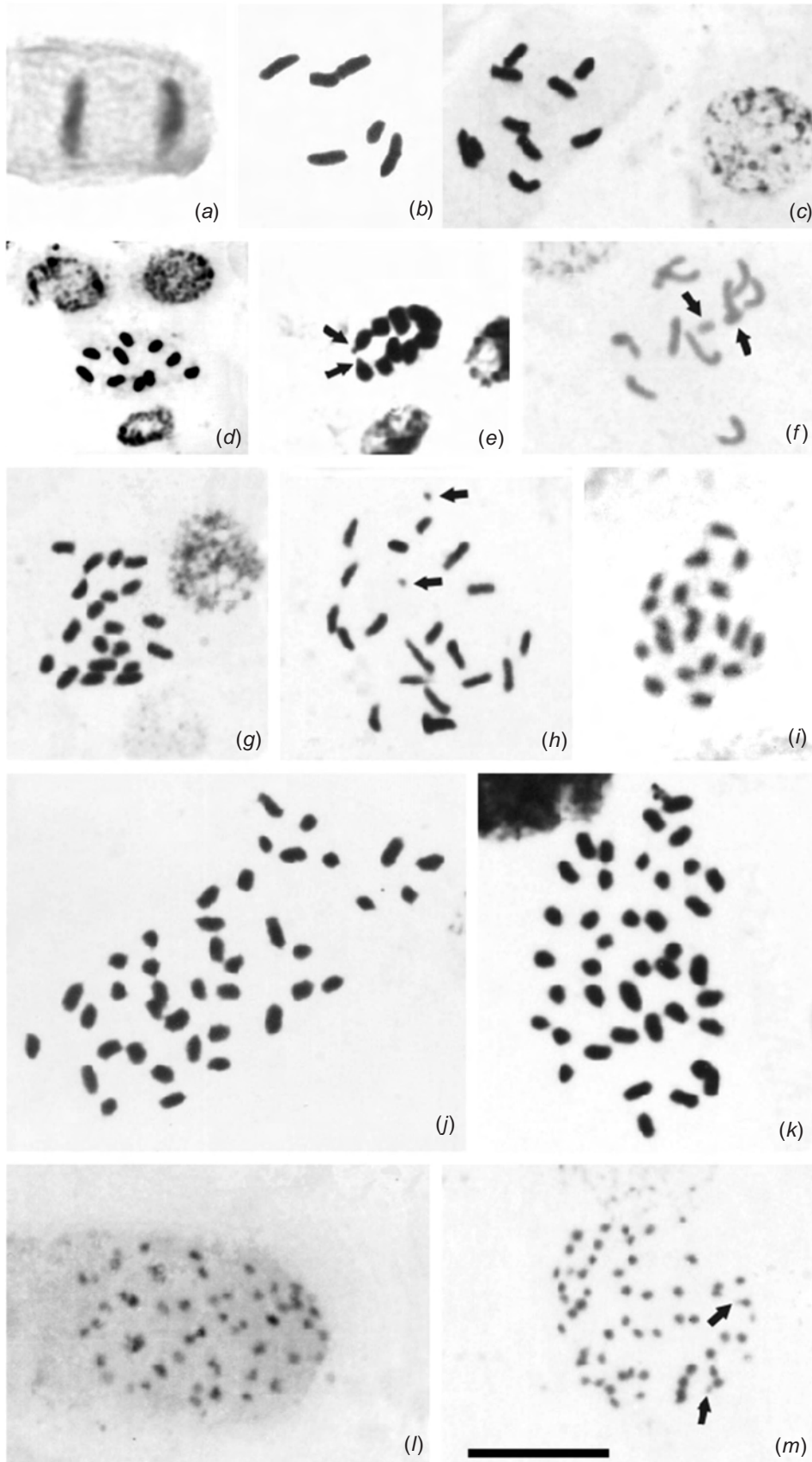
## Results and discussion

### *Karyotype features and cytotaxonomy*

The reduction in morphological structures of *Eleocharis* also reaches the chromosome level. Cyperaceae possess holocentric chromosomes, so that it is not possible to compare populations and species by the use of chromosome shape. Cytogenetical features, such as nucleolar constrictions, chromosome size and interphase nuclei types, can be useful to study many plant groups; however, this information is scarce in the literature on Cyperaceae. Hoshino (1987) reported that interphasic nuclei in *Eleocharis* possess diffuse chromatin with spherical bodies stained darkly and with differences in size. The nuclei described by Hoshino (1987), as well as those found in the present study, and those described by Vanzela *et al.* (2000) in *Rhynchospora*, are similar to the chromocentric interphase nuclei described by Kondo *et al.* (1994) in *Drosera* (Droseraceae), which also has holocentric chromosomes. Thus, the occurrence of chromocentric nuclei in all the studied species of *Eleocharis*, with minor differences in the chromocenter size (see Fig. 1c, d, g), suggests that this feature is not a useful cytotaxonomic tool to separate species and subgenera of *Eleocharis*; however, it indicates that besides the holokinetic chromosomes (Greilhuber 1995), the nucleus type may also be a synapomorphy of the Cyperaceae.

None of the species studied presented primary constrictions in the chromosomes and, additionally, the chromosomes migrate parallel to the equatorial plate axis at anaphase, as can be observed e.g. in *E. sellowiana* (Fig. 1a). The absence of Rabl organisation during separation of sister chromatids in Cyperaceae, was previously noted in *R. tenuis* (Vanzela *et al.* 1996), and confirmed recently by Guerra *et al.* (2005), after the detection of mitotic apparatus with anti- $\alpha$ -tubulin antibody. Several studies have shown the occurrence of holocentric chromosomes in *Eleocharis* (Strandhede 1965; Hoshino 1987; da Silva *et al.* 2005), reinforcing that the holokinetic condition of the chromosomes may be a synapomorphy of the family, as proposed by Greilhuber (1995).

**Fig. 1.** Conventional chromosome analysis in *Eleocharis*. (a) Anaphase in *Eleocharis sellowiana*, showing the parallel movement of the chromosomes at the equatorial plate; (b) metaphase with  $2n = 6$  in *E. subarticulata*; (c) metaphase with  $2n = 10$  in *E. maculosa*; (d) metaphase with  $2n = 10$  in *E. flavescens* (note the chromocentric interphase nuclei in c and d); (e) metaphase with  $2n = 10$  in *E. radicans* (arrows point to the terminal nucleolar constrictions (satellites)); (f) prometaphase with  $2n = 12$  in *E. minima*; two small chromosomes (arrows); (g) metaphase with  $2n = 20$  in *E. geniculata*; adjacent, a diffuse chromocentric nucleus; (h) prometaphase with  $2n = 20$  in *E. sellowiana*; arrows point to two satellites; metaphase in *E. montana* with (i)  $2n = 20$  and (j)  $2n = 40$ ; (k) metaphase with  $2n = 40$  in *E. elegans*; (l) metaphase with  $2n = \sim 54$  in *E. brasiliensis*; and (m) metaphase with  $2n = 54$  in *E. acutangula*; arrows point to two minor satellites. Scale bar = 10  $\mu\text{m}$ .





For eight of the species studied (Fig. 1f, h–l; Table 1), the chromosome number is documented here for the first time. Chromosome number for the other three (*E. acutangula*, *E. geniculata* and *E. subarticulata*) are confirmed here (Fig. 1b, g, m; Table 1). The number varied from  $2n=6$  in *E. subarticulata* (Fig. 1b) to  $2n=\sim 54$  in *E. brasiliensis* and  $2n=54$  in *E. acutangula* (Fig. 1l, m). *E. maculosa* (Fig. 1c), *E. flavescens* (Fig. 1d) and *E. radicans* (Fig. 1e) exhibited  $2n=10$ , whereas *E. geniculata* and *E. sellowiana* showed a duplicated chromosome number, with  $2n=20$  (Fig. 1g, h). An intermediate chromosome number,  $2n=12$ , was found in *E. minima* (Fig. 1f); however, in this species, two of the chromosomes are very small (arrows). Cytotypes were found in *E. montana* with  $2n=20$  and  $40$  (Fig. 1i, j). In this case, the chromosomes of the cytotype with  $2n=20$  varied from  $1.3$  to  $2.3\ \mu\text{m}$ , and those of the cytotype with  $2n=40$  varied from  $1.1$  to  $2.2\ \mu\text{m}$ . The total length of the complement was twice the normal length in the populations with  $2n=40$ . Significant morphological differences were not found between the two cytotypes of *E. montana*, suggesting that the polyploidisation to  $2n=40$  could be a recent event, since it does not yet affect the gross morphology. The chromosome number  $2n=40$  was also found in *E. elegans* (Fig. 1k). Another interesting case involves *E. sellowiana* and *E. flavescens*, two species in which the morphological characters, in some instances, are not enough to distinguish them. Some differences are the transversally elliptic stems in *E. flavescens* but circular in *E. sellowiana* (Faria 1998). Also, the upper portion of the distal sheath is not abruptly differentiated from the lower portion in *E. flavescens*, whereas in *E. sellowiana* it is abruptly differentiated (González-Elizondo *et al.* 2005). Populations of *E. sellowiana*, when slender or depauperate, may be morphologically very similar to and difficult to differentiate from *E. flavescens*. However, the karyotype of these species can be considered an additional micromorphological feature useful to taxonomy, since *E. sellowiana* possesses  $2n=20$  and *E. flavescens*  $2n=10$ .

Intraspecific variations, as those found here, are common in *Eleocharis* and some other Cyperaceae. Numerical variations were described by Bureš *et al.* (2004) in an extensive study of 78 European populations of *E. palustris* subsp. *palustris*. These authors found three prevalent cytotypes, namely, the diploid with  $2n=16$  and, in fewer samples, the symploid ( $2n=15$ ) and the mixoploid ( $2n=15$  and  $16$ ). Ohkawa *et al.* (2000) studied different populations of *Carex sociata* from Japan and found numbers varying from  $2n=40$  to  $44$ , with prevalence of  $2n=42$  and  $44$ . Such variations have also been found in genera such as *Fimbristylis*: *F. bisumbellata* and *F. dichotoma*, both with populations with  $2n=10$  and  $20$ , and *F. falcata* with  $n=11$  and  $22$  (Bir *et al.* 1992). In the genus *Rhynchospora*, *R. ciliata* was treated as a subspecies of *R. nervosa*; however, *R. ciliata* showed  $2n=10$  and *R. nervosa*  $2n=30$ ; thus, Luceño *et al.* (1998) proposed that they could be considered as two different species. Polyploid and diploid cytotypes have been found in *E. geniculata*, with  $2n=10$  and  $20$ , and *E. atropurpurea* with  $2n=20$  and  $21$  (Nijalingappa 1973). The study that described the largest numerical variations among related species was published by Strandhede (1965). This author showed that in

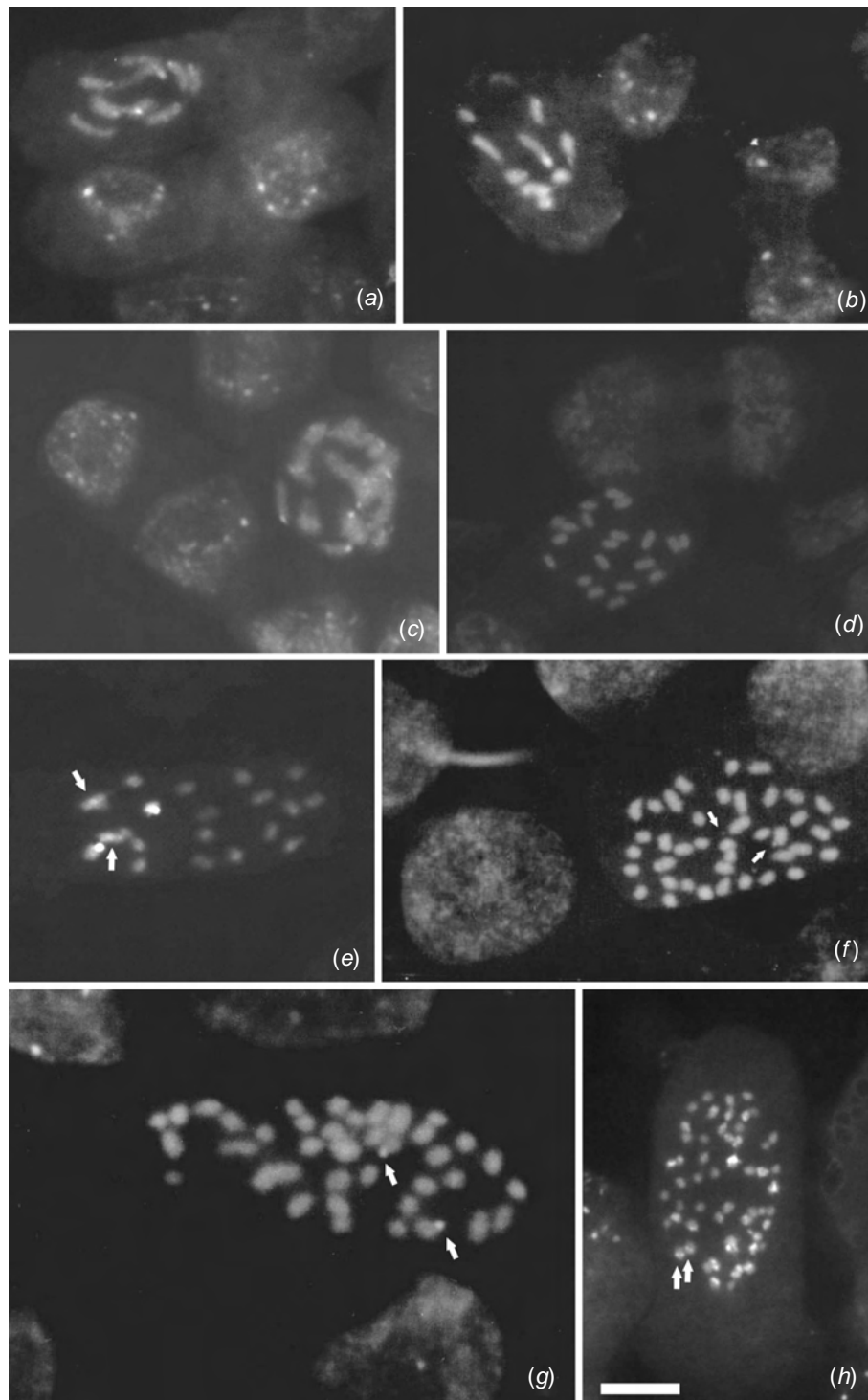
*E.* subsp. *Eleocharis*, *E. mamillata* has  $2n=16$ , *E. palustris* subsp. *palustris* has  $2n=15$  and  $16$ , *E. palustris* subsp. *vulgaris* showed  $2n=36$ ,  $38$ ,  $39$  and  $42$ , and *E. uniglumis*  $2n=46$  and  $2n=74$  to  $82$ .

Most of the chromosome numbers found in *Eleocharis* are multiples of  $x=5$ , which is in line with the proposition of Löve *et al.* (1957) about Cyperaceae. This basic number is common in some close genera, such as *Bulbostylis*, *Fimbristylis* (Bir *et al.* 1992) and *Abildgaardia* subsp. (André L. L. Vanzela 2006, unpubl. data), but also in other, more distant genera, such as *Rhynchospora* (Luceño *et al.* 1998). Secondary basic numbers have also been detected in *Eleocharis*; however, these are probably derived from structural changes. *E. subarticulata* with  $2n=6$  is a good example. In this case, there was a reduction owing to multiple translocations (da Silva *et al.* 2005). Reduction in the number by fusion of holocentric chromosomes was reported in *R. tenuis*, with  $2n=4$  (Vanzela *et al.* 1996) and *Fimbristylis umbellaris* with  $2n=6$  (Rath and Patnaik 1978). If we consider  $x=5$  as the primary basic number of *Eleocharis*, it is possible that the two minor chromosomes found in *E. minima* with  $2n=12$  appeared by fission (agmatoploidy) and the numbers  $2n=\sim 54$  found in *E. brasiliensis* and  $2n=54$  found in *E. acutangula* (Fig. 1l, m) probably originated from agmatoploidy followed by or preceding polyploidy. It is also possible that the high chromosome number in these species has appeared by widespread chromosome fission and not only by polyploidy. A similar event was reported by Nordenskiöld (1951), who observed that in some polyploid species of *Luzula* (Juncaceae), the holocentric chromosomes seemed to be half the size of those of the diploid species. An increase in the chromosome number resulting from agmatoploidy has been described in several genera of Cyperaceae. As examples, there are numerical variations in genera close to *Eleocharis*, such as *Schoenoplectus* with  $2n=42$ ,  $44$  and  $78$  (Jankun 1989; Löve and Löve 1981) and *Scirpus* with  $2n=20$ ,  $31$ ,  $32$ ,  $34$ ,  $37$ ,  $48$ ,  $50$ ,  $56$ ,  $60$  and  $62$  (Heiser 1979; Harriman 1981; Löve and Löve 1981; Pignotti and Fiorini 1998; Subramanian 1988).

Chromosome sizes of the species studied are shown in Table 1. Hoshino (1987) proposed the existence of the following three karyotype variations in *Eleocharis* in relation to the chromosome size: (i) bimodal variation, with two groups of chromosomes of distinctly different sizes, (ii) gradual variation, where the chromosomal size decreases gradually and (iii) homogeneous variation, where all the chromosomes are similar, and very small. Yano *et al.* (2004) studied several Japanese species of *Eleocharis* and suggested that *Eleocharis* can be divided into two groups according to chromosome sizes. The first includes species with very small chromosomes ( $<1.1\ \mu\text{m}$  in length), and the second includes species with chromosome sizes ranging from  $1.4$  to  $4.3\ \mu\text{m}$ . The chromosome measurements made in the present study corroborate the analyses by Hoshino (1987), and partially corroborate the results of Yano *et al.* (2004); the species of subgenus *Limnochloa* (*E. acutangula* and *E. brasiliensis*) have many and smaller chromosomes ( $1.2$ – $0.7\ \mu\text{m}$ ), whereas size is variable in the species of the subgenera *Eleocharis* and *Scirpidium*, ranging from  $4.1$

to 1.2  $\mu\text{m}$  (and even 0.9  $\mu\text{m}$  in the small chromosomes of *E. minima*). Species of subgenera *Eleocharis* and *Scirpidium* exhibit a bimodal pattern of karyotype, where the numbers

form a gradual, incremental sequence of numbers. Our findings are consistent with the classification system proposed by González-Elizondo and Peterson (1997) and with the



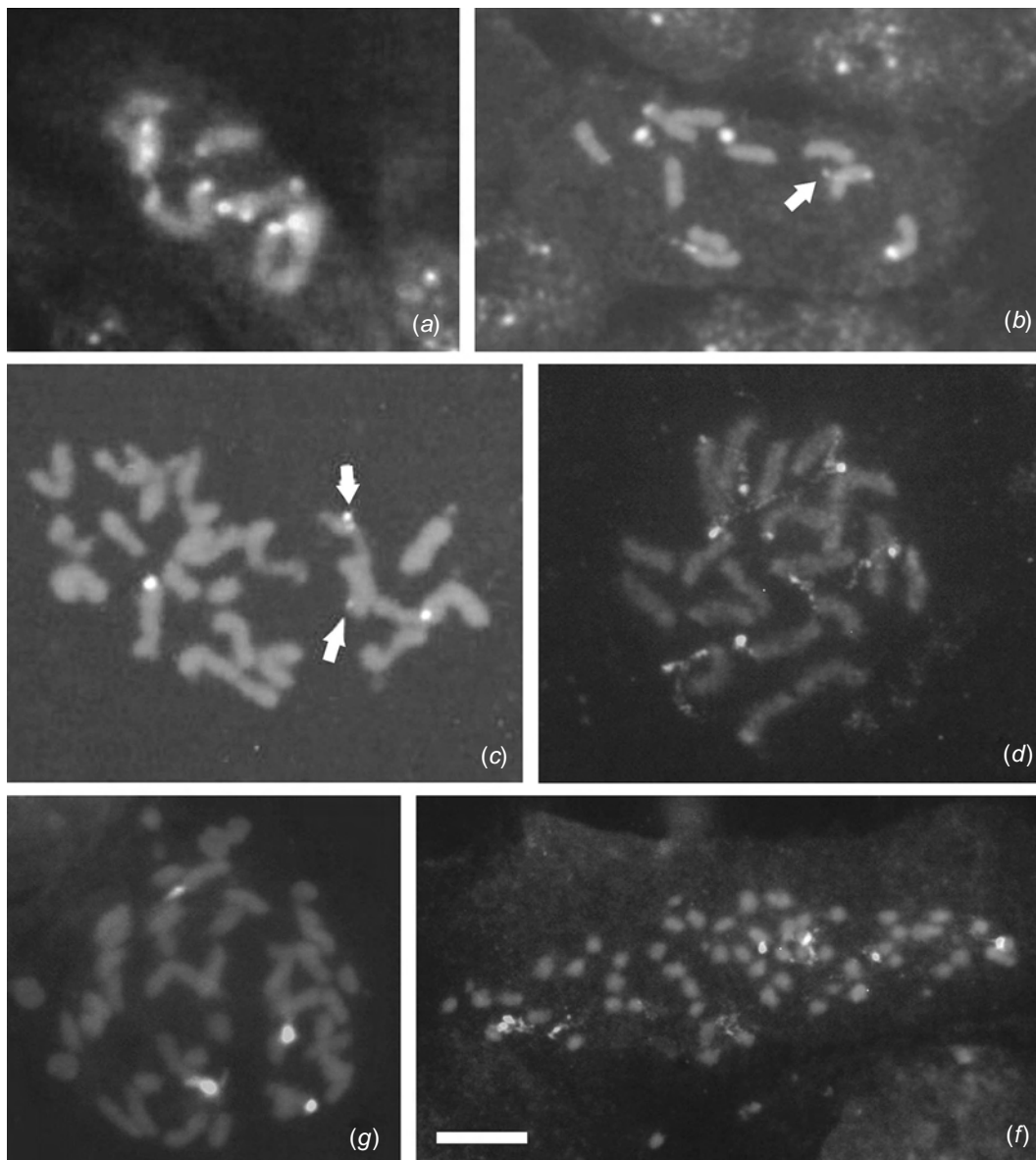
**Fig. 2.** C-CMA<sub>3</sub>/DAPI banding in *Eleocharis*. (a) *Eleocharis flavescens* ( $2n = 10$ ) with four terminal CMA<sub>3</sub> blocks; (b) *E. maculosa* ( $2n = 10$ ) with four terminal CMA<sub>3</sub> blocks; (c) *E. sellowiana* ( $2n = 20$ ) with about 10 terminal C-CMA<sub>3</sub> blocks; (d) C- DAPI banding in *E. sellowiana*; note the absence of DAPI blocks; (e) *E. geniculata* ( $2n = 20$ ), showing two larger terminal C-CMA<sub>3</sub> blocks and two smaller and interstitial (arrows); (f) *E. elegans* ( $2n = 40$ ); arrows indicate the two tenuous and terminal C-CMA<sub>3</sub> blocks; note the absence of signals in the nuclei; (g) *E. montana* ( $2n = 40$ ) with two terminals C-CMA<sub>3</sub> blocks (arrows); and (h) *E. acutangula* ( $2n = 54$ ) with  $\sim 20$  terminal and few interstitial C-CMA<sub>3</sub> bands; the arrows point to two interstitial blocks. Scale bar = 5  $\mu\text{m}$ .

phylogenetical analysis based on ITS proposed by Roalson and Friar (2000).

#### *Repetitive DNA and chromosome organisation*

The C-CMA<sub>3</sub>/DAPI banding showed that the studied species have no DAPI bands (Fig. 2*d*), suggesting that AT-rich sequences seem not to be important in the karyotype organisation. However, most of the species showed fine terminal C-CMA<sub>3</sub> bands (GC-rich blocks), with low fluorescence intensity, but varying in the number and size of the bands. *E. flavescens* and *E. maculosa*, both with  $2n = 10$ , exhibited four C-CMA<sub>3</sub> signals, two signals being slightly larger and two smaller (Fig. 2*a, b*). *E. sellowiana*

with  $2n = 20$  exhibited  $\sim 10$  C-CMA<sub>3</sub> blocks, eight being more conspicuous and two fine (Fig. 2*c*). *E. geniculata* with  $2n = 20$  showed four C-CMA<sub>3</sub> blocks, two being larger and terminal and two smaller and interstitial. Interestingly, these interstitial blocks were found in the largest pair (Fig. 2*e*). *E. elegans* and *E. montana*, both with  $2n = 40$ , showed tenuous C-CMA<sub>3</sub><sup>+</sup> bands always in one chromosome pair (Fig. 2*f, g*). Interestingly, *E. acutangula*, the species with the smallest chromosomes, presented the largest number of C-CMA<sub>3</sub><sup>+</sup> bands, with  $\sim 20$  interstitial and terminal bands (Fig. 2*h*). In spite of the very small number of treated species for the chromosome banding, these data indicate that heterochromatin rich in GC is very important



**Fig. 3.** FISH with 45S rDNA probe in species of *Eleocharis*. (a) *E. flavescens* ( $2n = 10$ ) with 10 rDNA sites; (b) *E. maculosa* ( $2n = 10$ ), showing six rDNA sites, two being minor and four major; the arrows point to the minor sites; note also the spread out aspect at prometaphases; (c) *E. geniculata* ( $2n = 20$ ), showing four hybridisation sites; arrows point to the two minor rDNA sites; (d) *E. sellowiana* ( $2n = 20$ ), exhibiting eight rDNA sites; note the spread out aspect of hybridisation sites; (e) *E. montana* with four rDNA sites; and (f) *E. acutangula* ( $2n = 54$ ), showing eight 45S rDNA sites. Scale bar = 5  $\mu$ m.

in chromosome organisation in *Eleocharis*, whereas AT-rich segments represent a repetitive DNA family that perhaps appears sporadically in a few species, with a punctual importance. Sheikh and Kondo (1995) used chromosome banding to study the karyotypical differences in *Drosera* (Droseraceae), another group containing holocentric chromosomes. These authors found that in 12 species, the bands appeared preferentially in the terminal chromosome positions, but with accumulation of CMA-positive and not DAPI-positive bands, as observed here in *Eleocharis*. In contrast to our results, there are two published examples of GC- and AT-rich heterochromatin accumulation. In the first case, Vanzela and Guerra (2000) described three heterochromatin types in the genus *Rhynchospora* (Cyperaceae), i.e. CMA<sub>3</sub><sup>+</sup>/DAPI<sup>-</sup>, CMA<sub>3</sub><sup>+</sup>/DAPI<sup>+</sup>, and CMA<sub>3</sub><sup>-</sup>/DAPI<sup>+</sup>, which varied in size and position; however, only the karyotype of *R. globosa* was differentiated owing to accumulation of AT-rich blocks. In the second case, Guerra and García (2004) described a large amount of heterochromatin (more AT- than GC-rich bands) in *Cuscuta approximata* (Cuscutaceae). In fact, the accumulation and distribution of heterochromatin in holocentric chromosomes seems to be very similar to that known for monocentric chromosomes (Guerra 2000).

The common association among terminal GC-rich blocks with nucleolar organiser regions was previously shown in other Cyperaceae, including *Carex* (Greilhuber 1995) and *Rhynchospora* (Vanzela and Guerra 2000). However, on the basis of the species investigated here, the association between GC-rich heterochromatin and NOR is not a rule. Thus, these data suggest the occurrence of at least two repetitive DNA families in *Eleocharis*: (1) C-CMA<sub>3</sub><sup>+</sup> bands NOR associated and (2) C-CMA<sub>3</sub><sup>+</sup> not NOR associated. Nucleolar constrictions are not frequently visualised on holocentric chromosomes, and following this tendency, only a few chromosomes with satellites (terminal NORs) were seen in *Eleocharis*. These terminal constrictions were found in *E. radicans* ( $2n = 10$ ), *E. sellowiana* with  $2n = 20$  and *E. acutangula* with  $2n = 54$  (Fig. 1e, h, m). Similar constrictions were also described by Hoshino (1987) in *Eleocharis* and by Vanzela *et al.* (1996) and Luceño *et al.* (1998) in *Rhynchospora*.

To confirm the number of NORs, FISH with 45S rDNA probe was performed. The pT<sub>71</sub> probe localised hybridisation sites always at the chromosome ends, as previously reported by Vanzela *et al.* (1998) in *Rhynchospora*, by Furuta and Hoshino (1999) in *E. mamillata* var. *cyclocarpa* and by Guerra and García (2004) in *C. approximata* (Cuscutaceae). These latter authors hybridised chromosomes with 18S rDNA probes and found 12 terminal hybridisation sites, with two small chromosomes with rDNA sites at both extremities. The results of FISH with the 45S rDNA probe, according to our study, also showed multiple and terminal hybridisation sites, and that the differences in hybridisation site number were independent of the ploidy level. The hybridisation sites appear spread out at prometaphase and metaphase, hindering determination of the number of sites. Ten 45S rDNA sites were found in *E. flavescens* with  $2n = 10$  (Fig. 3a), in which only four are associated to the GC-rich heterochromatin. In *E. maculosa* ( $2n = 10$ ), six rDNA sites were observed, two being minor and four major (Fig. 3b), and these four are associated with GC-rich blocks. *E. geniculata* ( $2n = 20$ )

showed four hybridisation sites (Fig. 3c). *E. sellowiana* ( $2n = 20$ ) showed eight rDNA sites (Fig. 3d), and all associated with GC-rich blocks. *E. montana* ( $2n = 40$ ) exhibited four rDNA sites (Fig. 3e), with two associated with GC-rich heterochromatin. *E. acutangula* ( $2n = 54$ ) showed eight 45S rDNA sites (Fig. 3f) and a larger number of GC-rich blocks. The multiplicity of 45S rDNA sites and the occurrence of two heterochromatin types (GC-rich associated and not associated with NOR) observed in *Eleocharis*, were also reported in *Rhynchospora* by Vanzela *et al.* (1998), suggesting a cytogenetical feature common for Cyperaceae.

In conclusion, these results show that despite of the reduction in the number of morphological and cytogenetical features (considering the absence of primary constrictions), the karyotypes of *Eleocharis* accumulate differences in the size and number of chromosomes between species and populations as well as in the size and number of GC-rich bands and 45S rDNA sites. Hence, cytogenetics can be considered a good tool for taxonomic and evolutionary studies in Cyperaceae.

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**Capítulo 3**

Distribution of 45S and 5S rDNA sites in 23 species of  
*Eleocharis* (Cyperaceae)

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Title: Distribution of 45S and 5S rDNA sites in 23 species of *Eleocharis* (Cyperaceae)

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Keywords: FISH, holocentric chromosomes, rDNA distribution, amplification and location

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**Abstract:** Studies of rDNA location in holocentric chromosomes of Cyperaceae are scarce, but a few reports have indicated the predominance of 45S rDNA in terminal positions, multiple numbers of sites and a decondensed state of hybridization region in prometaphase/metaphase. To increase the knowledge of the number 45S and 5S rDNA sites and distribution in holocentric chromosomes of Cyperaceae, 23 Brazilian species of *Eleocharis* were studied. FISH showed 45S rDNA signals always located in terminal regions, which varied from two (*E. bonariensis* with  $2n = 20$ ) to ten (*E. flavescens* with  $2n = 10$  and *E. laeviglumis* with  $2n = 60$ ). The 5S rDNA showed less variation, with 16 species exhibiting two sites and 7 species with four sites, preferentially in terminal positions, except for 4 species (*E. subarticulata*, *E. flavescens*, *E. sellowiana* and *E. geniculata*) that showed interstitial sites. Results are discussed in order to understand the prevalence of terminal rDNA sites, the mechanisms involved in the interstitial allocation of 5S rDNA sites and the events of amplification and dispersion of 45S rDNA.

1 **Distribution of 45S and 5S rDNA sites in 23 species of**  
2 ***Eleocharis* (Cyperaceae)**

3

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10

11 **Abstract**

12 Studies of rDNA location in holocentric chromosomes of Cyperaceae are scarce, but a  
13 few reports have indicated the predominance of 45S rDNA in terminal positions,  
14 multiple numbers of sites and a decondensed state of hybridization region in  
15 prometaphase/metaphase. To increase the knowledge of the number 45S and 5S rDNA  
16 sites and distribution in holocentric chromosomes of Cyperaceae, 23 Brazilian species  
17 of *Eleocharis* were studied. FISH showed 45S rDNA signals always located in terminal  
18 regions, which varied from two (*E. bonariensis* with  $2n = 20$ ) to ten (*E. flavescens* with  
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20 species exhibiting two sites and 7 species with four sites, preferentially in terminal  
21 positions, except for 4 species (*E. subarticulata*, *E. flavescens*, *E. sellowiana* and *E.*  
22 *geniculata*) that showed interstitial sites. Results are discussed in order to understand  
23 the prevalence of terminal rDNA sites, the mechanisms involved in the interstitial  
24 allocation of 5S rDNA sites and the events of amplification and dispersion of 45S  
25 rDNA.



1 **Key words:** FISH, holocentric chromosomes, rDNA distribution, amplification and  
2 location

3

#### 4 **Introduction**

5         The rRNA genes occur as moderately repetitive DNA families on eukaryote  
6 genomes. The 45S rDNA of plants, as well as in other organisms, appears as repeated  
7 and clustered segments at particular chromosomal positions. These segments  
8 predominantly occupy the chromosome ends (Furuta and Kondo 1999; Rego et al.  
9 2009), ranging in the number of sites among and within species (Vanzela et al. 1998; De  
10 Melo and Guerra 2003; Da Silva et al. 2008a; Raskina et al. 2008). The 5S rDNA is also  
11 organized in repeated and clustered segments, occurring in most cases in distinct  
12 chromosome positions of those occupied by 45S rDNA (Moscone et al. 1999).  
13 Although these segments vary in the number of sites and chromosome pairs in different  
14 organisms, they tend to occupy similar chromosome and position in closely related  
15 species, as in *Aristolochia* (Berjano et al. 2009).

16         Plant holocentric chromosomes were reported in all representatives of the  
17 families Juncaceae Juss. and Cyperaceae Juss. (Bokhari and Godward 1980; Roalson et  
18 al. 2008), in some species of genus *Drosera* - Droseraceae and subgenus *Cuscuta* -  
19 Cuscutaceae. However, there are two isolated species, *Chionographis japonica* -  
20 Liliaceae and *Myristica fragrans* Myristicaceae, which present this chromosome type  
21 (Flach 1966; Tanaka and Tanaka 1977; Sheikh et al. 1995; García 2001). The locations  
22 of rDNA sites have only been determined in a few studies. Multiple 45S rDNA sites  
23 were detected in nine species of *Rhynchospora* (Vanzela et al. 1998, 2003) and seven  
24 species of *Eleocharis* (Da Silva et al. 2005, 2008a, b), both of the Cyperaceae family.  
25 Hoshi (1995) and Furuta and Kondo (1999) also reported multiple and terminal rDNA

1 sites in *Drosera*. However, in *Cuscuta approximata* one pair of 45S rDNA and three of  
2 5S rDNA sites were reported (Guerra and García 2004). In spite of general tendency  
3 towards a terminal location of 45S rDNA, the 5S rDNA was reported preferentially in  
4 interstitial regions in plants and animals, and in *Eleocharis* the interstitial occurrence of  
5 5S rDNA was associated to chromosome rearrangement (Da Silva et al. 2008b).

6 Studies about the amount and distribution of rDNA sites in plants with  
7 holocentric chromosomes are scarce. Cyperaceae family possess approximately 5,500  
8 species (Govaerts et al. 2007), but less than 1% of studied species possess rDNA as  
9 probed by FISH. These few reports do not discuss the location and amplification of  
10 rDNA based in the models, as is commonly done in organisms with monocentric  
11 chromosomes. The aim of this study was to localize the 45S and 5S rDNA sites using  
12 double-FISH in 23 Brazilian species of *Eleocharis* containing different chromosome  
13 number and size. Data were discussed to assess the karyotype diversity involving the  
14 rDNA sites and to understand the possible mechanisms that are operating in the rDNA  
15 amplification and distribution in organisms with holocentric chromosomes.

16

## 17 **Material and Methods**

18 Three individuals of each of 23 species of *Eleocharis* were collected in different  
19 Brazilian states (Table 1). Samples were cultivated in a greenhouse at the Laboratório  
20 de Biodiversidade e Restauração de Ecossistemas (LABRE) at Universidade Estadual  
21 de Londrina, Paraná, Brazil. Vouchers were deposited at the ICN herbarium of the  
22 Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil (Table 1). Metaphase  
23 cells were obtained from root tips pre-treated with 2mM 8-hydroxyquinolin for 24 h,  
24 fixed in ethanol:acetic acid (3:1, v:v) for 24 h, and stored at -20 °C. Samples were

1 softened in 4% cellulase plus 40% pectinase (w:v) at 37 °C for 4 h, squashed in a drop  
2 of 60% acetic acid, and the cover slips were removed after freezing in liquid nitrogen.

3 Fluorescent in situ hybridization (FISH) was performed as described in Da Silva  
4 et al. (2008a). The 45S rDNA probe (pTa71) from *Triticum aestivum* was labeled with  
5 digoxigenin-11-dUTP and the 5S rDNA probe from *Rhynchospora pubera* (Angeles  
6 Cuadrado, unpublished) was labeled with biotin-14-dATP, both by nick translation.  
7 Probes were simultaneously utilized for FISH in a mixture of 30 µL containing 100%  
8 formamide (15 µL), 50% polyethylene glycol (6 µL), 20× SSC (3 µL), 100 ng of calf  
9 thymus DNA (1 µL), 10% SDS (1 µL), and 200 ng of probes (4 µL each). The material  
10 was denatured at 90 °C for 10 min and hybridization was performed at 37 °C overnight  
11 in a humidified chamber. Post-hybridization washes were carried out with 70%  
12 stringency, using baths with SSC buffer.

13 The probes were simultaneously detected with avidin-FITC and anti-  
14 digoxigenin-rhodamine conjugates. The post-detection washes were performed in 4×  
15 SSC/0.2% Tween 20, all at room temperature. Slides were mounted with 25 µL of  
16 DABCO solution, composed of glycerol (90%), 1,4-diaza-bicyclo(2,2,2)-octane (2.3%),  
17 20 mM TrisHCl, pH 8.0 (2%), 2.5 mM MgCl<sub>2</sub> (4%) and distilled water (1.7%), plus 4  
18 µl of 2 µg.mL<sup>-1</sup> DAPI.

19 All the chromosome images were acquired separately without color (grayscale)  
20 with a Leica DM 4500 B microscope coupled with a DFC 300FX camera and  
21 overlapped with red color for DAPI, white for rhodamine (45S rDNA) and greenish-  
22 yellow (5S rDNA) for FITC, using the Leica IM50 4.0 software. The images were  
23 optimized for best contrast and brightness with Adobe Photoshop CS3 version 10.0  
24 software.

1

2 **Results**

3         Twenty-three species were analyzed (among these, 15 belonged to the subgenus  
4 *Eleocharis*, two to *Scirpidium* and six to *Limnochloa*) according to González-Elizondo  
5 and Peterson (1997) (Table 1). The number of 45S rDNA sites varied from two to ten  
6 independent of chromosome numbers and subgenus. In contrast, terminal location of  
7 45S rDNA was constant in all species. The 5S rDNA probe showed a minor variation  
8 when compared to 45S rDNA, ranging from two to four chromosome sites which were  
9 terminal in 19 species and interstitial in four others (Table 1). The rDNA hybridization  
10 signals also varied in size among homologous, as observed in *E. niederleinii* (Fig. 1c),  
11 *E. montana* (Fig. 1g), *E. debilis* (Fig. 2f) and *E. obtusetrigona* (Fig. 3b). In all cases, the  
12 45S rDNA appeared diffused/decondensed and different of the 5S rDNA probed, even  
13 when they were located in the terminal region.

14         The representatives of subgenus *Eleocharis* showed no correspondence between  
15 polyploidy level and rDNA site numbers. *Eleocharis flavescens*, with  $2n = 10$ , exhibited  
16 signals of 45S rDNA in all chromosomes of the complement and only one interstitial 5S  
17 rDNA signals located in pair 1 (Fig. 1k), while *E. nana* with  $2n = 20$ , showed two  
18 terminal signals of each of 45S and 5S rDNA (Fig. 1b). Two interstitial signals of 5S  
19 rDNA were also found in *E. subarticulata* ( $2n = 6$ ), *E. sellowiana* and *E. geniculata*,  
20 but, in these latter two species, karyotypes with  $2n = 10$  and 20 were found. These  
21 species also showed variations in the number of terminal 45S rDNA sites, being four in  
22 *E. subarticulata* (Fig. 1f), in the diploid and polyploid of *E. geniculata* (Figs. 2c and d),  
23 and in the diploid form of *E. sellowiana* and eight in the polyploid form of *E.*  
24 *sellowiana* (Figs. 2a and b). *Eleocharis contracta* (Fig. 1a), *E. niederleinii* (Fig. 1c), *E.*  
25 *loefgreniana* (Fig. 1d), *E. viridans* (Fig. 1e), *E. montana* (Fig. 1g), *E. minima* (Fig. 1h),

1 *E. filiculmis* (Fig. 1i), *E. capillacea* (Fig. 1j), *E. maculosa* (Fig. 1l) and *E. debilis* (Fig.  
2 2f) exhibited distinct chromosome numbers but equal rDNA site numbers of rDNA  
3 sites, being four signals to 45S rDNA and two to 5S, always in terminal position (Table  
4 1).

5 The two species of the subgenus *Scirpidium* varied in number and position of  
6 rDNA sites. *Eleocharis bonariensis* with  $2n = 20$  showed four 45S rDNA sites and  
7 four signals of 5S rDNA, always in terminal positions (Fig. 2g), while *Eleocharis* sp.,  
8 with  $2n = 10$  (a new species cited in Da Silva et al. 2010, but yet undetermined) showed  
9 six chromosomes with 45S rDNA signals and another two with 5S rDNA, also always  
10 in terminal positions (Fig. 2e).

11 Six species of the subgenus *Limnochloa* were studied. These species showed  
12 different chromosome number (40, 50, 52, 54 and 60) and exhibited the smallest  
13 chromosomes of the genus. Four 5S rDNA terminal sites were found in all species but  
14 the number of terminal 45S rDNA sites varied. *Eleocharis obtusetrigona*, *E. acutangula*  
15 and *E. interstincta* presented eight hybridization signals (Figs. 3b, d and e), while *E.*  
16 *plicarhachis*, *E. liesneri* and *E. laeviglumis* showed 10 sites (Figs. 3a, c and f).

17

## 18 **Discussion**

19 Cyperaceae is marked by a high variability in chromosome number (Roalson  
20 2008), provoked mainly by events of symploidy (fusion), agmatoploidy (fission) and  
21 polyploidy (Håkanson 1958; Vanzela et al. 2000; Yano et al. 2004; Hipp et al. 2009).  
22 The basic chromosome number,  $x = 5$ , proposed by Löve et al. (1957) for Cyperaceae is  
23 accepted by several authors (Vanzela et al. 1998; Yano et al. 2004; Da Silva et al.  
24 2008a), even considering the high numeric variability. In the context of 45S rDNA sites,  
25 it would be natural to expect that the karyotypes with few chromosomes would also

1 present few rDNA sites. However, we found species with  $2n = 10$  with four signals (the  
2 majority of species), six signals (*Eleocharis* sp.) and signals in all chromosomes, such  
3 as in *E. flavescens*. Multiple hybridization sites were previously reported to *Eleocharis*  
4 *flavescens* (Da Silva et al. 2008a), as well as in some species of *Rhynchospora* (Vanzela  
5 et al. 1998) and *Drosera* (Hoshi 1995).

6 In this sense, the polyploids should have the double the number of sites in  
7 relation to their diploid representatives, as reported in *Rhynchospora tenuis* with  $2n = 4$   
8 and  $2n = 8$ , containing two and four 45S rDNA sites, respectively (Vanzela et al. 2003).  
9 This was also observed in *E. sellowiana* (with four and eight 45S rDNA signals). On the  
10 other hand, *Eleocharis geniculata* exhibited the same number of sites in both  $2n = 10$   
11 and  $2n = 20$ . The remaining polyploid species with  $2n = 20, 30$  and  $40$  showed two and  
12 four 45S rDNA signals, as did some diploid species (see Table 1). Fissions could  
13 explain the relationship between the reduction of 45S rDNA sites number and increase  
14 of chromosome number, but, there is no a conclusive evidence of such events in these  
15 mentioned cases. Thus, the most likely explanation would a loss of sites due to the  
16 diploidization process, as proposed by Leitch and Bennett (1997). Similar events were  
17 reported in *Passiflora* (De Melo and Guerra 2003).

18 Species of subgenus *Limnochloa* presented karyotypes with many and small  
19 chromosomes and little variation in size among them. Yano et al. (2004) and Da Silva et  
20 al. (2008a) have proposed that these karyotypes may have been generated by  
21 chromosome fission, but it was not confirmed in the later study of Da Silva et al.  
22 (2010). We found here that karyotypes of *Limnochloa* had multiple 45S rDNA sites  
23 (eight and ten). When we compared the FISH data in relation to karyotype organization,  
24 as chromosome number and symmetry (see Da Silva et al. 2010), regardless of  
25 systematic arrangement, it was possible to detect that the species with more symmetrical

1 karyotype, as in *E. interstincta* (*Limnochloa*), *Eleocharis* sp. (*Scirpidium*), *E. flavescens*  
2 and *E. sellowiana* (both of subgenus *Eleocharis*), exhibited a greater number of 45S  
3 rDNA sites. The karyotypes with a greater difference in chromosome sizes, i.e. more  
4 asymmetric, as *E. montana*, *E. nana* and *E. viridians* (all of subgenus *Eleocharis*),  
5 showed lower 45S rDNA sites.

6 Cases of multiple 45S rDNA sites have been described in both plants with  
7 holocentric chromosomes (Vanzela et al. 1998) and in plants with monocentric ones  
8 (Hasterok and Maluszynska 2000; Lim et al. 2001; Kwon and Kim 2009). Some  
9 mechanisms of amplification/dispersion of repetitive DNA have been proposed or  
10 mentioned for the monocentric system: i) rDNA sites mobility is associated with co-  
11 location and activity of transposable elements (Shubert and Wobus 1985; Raskina et al.  
12 2008) and ii) equilocal dispersion of heterochromatin and rDNA (Schweizer and Loidl  
13 1987; Pederson and Linde-Laursen 1994, respectively). In relation to first model, there  
14 are no reports in the literature involving the distribution of transposable elements in  
15 plant holocentric chromosomes. For the second model, however, Guerra (2000)  
16 interpreted that the equidistribution of repetitive DNA may depend on some structural  
17 or functional similarity of each chromosomal region. In this sense the polarization of  
18 rDNA sites observed in the interphase some *Rhynchospora* species might be due to an  
19 association of active sites building a single nucleolus (Vanzela et al. 1998). Similarly,  
20 the grouping of 45S rDNA regions depending on the NOR activity could contribute to  
21 the accumulation/dispersion of 45S rDNA segments to the chromosome ends with  
22 similar size. This could explain the occurrence of multiple rDNA sites preferably in  
23 karyotypes whose chromosomes have the same size (symmetrical). Idiograms and  
24 chromosome measurements showing the karyotype symmetry/asymmetry are detailed in  
25 Da Silva et al (2010).

1           The 5S rDNA probe hybridized in different regions of the 45S rDNA sites. The  
2 occurrence of 45S (terminal) and 5S rDNA (interstitial) sites in the same chromosome  
3 pair was found in *E. flavescens*, precisely because the 45S rDNA sites were distributed  
4 in the terminal regions of all chromosomes. The 5S hybridization signals were also  
5 detected in interstitial positions in *E. sellowiana*, *E. geniculata*, *E. subarticulata* and *E.*  
6 *maculosa*, but, in last two species the karyotypes with  $2n = 6$  and  $2n = 8$ , 7 and 6 were  
7 originated after chromosome rearrangements (Da Silva et al. 2005, 2008, respectively),  
8 and could support a reasonable explanation for the interstitial positioning of 5S rDNA.

9           The scientific advance of this article was the comparative analysis in a large  
10 number of species, i. e. about 30% of the Brazilian *Eleocharis*, with representatives of  
11 three subgenera. Our study showed that the 45S rDNA is more variable in sites number  
12 than 5S, but it could not state why the 45S sites never appeared in interstitial positions,  
13 as occurred with the 5S, since rearrangements are very common in the karyotype  
14 differentiation of Cyperaceae. Besides, it was unclear why the events that favor the  
15 multiplication and dispersion of 45S rDNA did not act in the same direction for 5S  
16 rDNA segments. In the future, efforts should be directed towards the investigation of  
17 the following aspects: (i) relationships between the location of 45S and 5S rDNA sites  
18 in other Cyperaceae groups, (ii) functionality of rDNA sites and (iii) relationships  
19 between rDNA segments detected by FISH, pseudogenes and transposable elements.

20

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24



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4

## 5 Legends

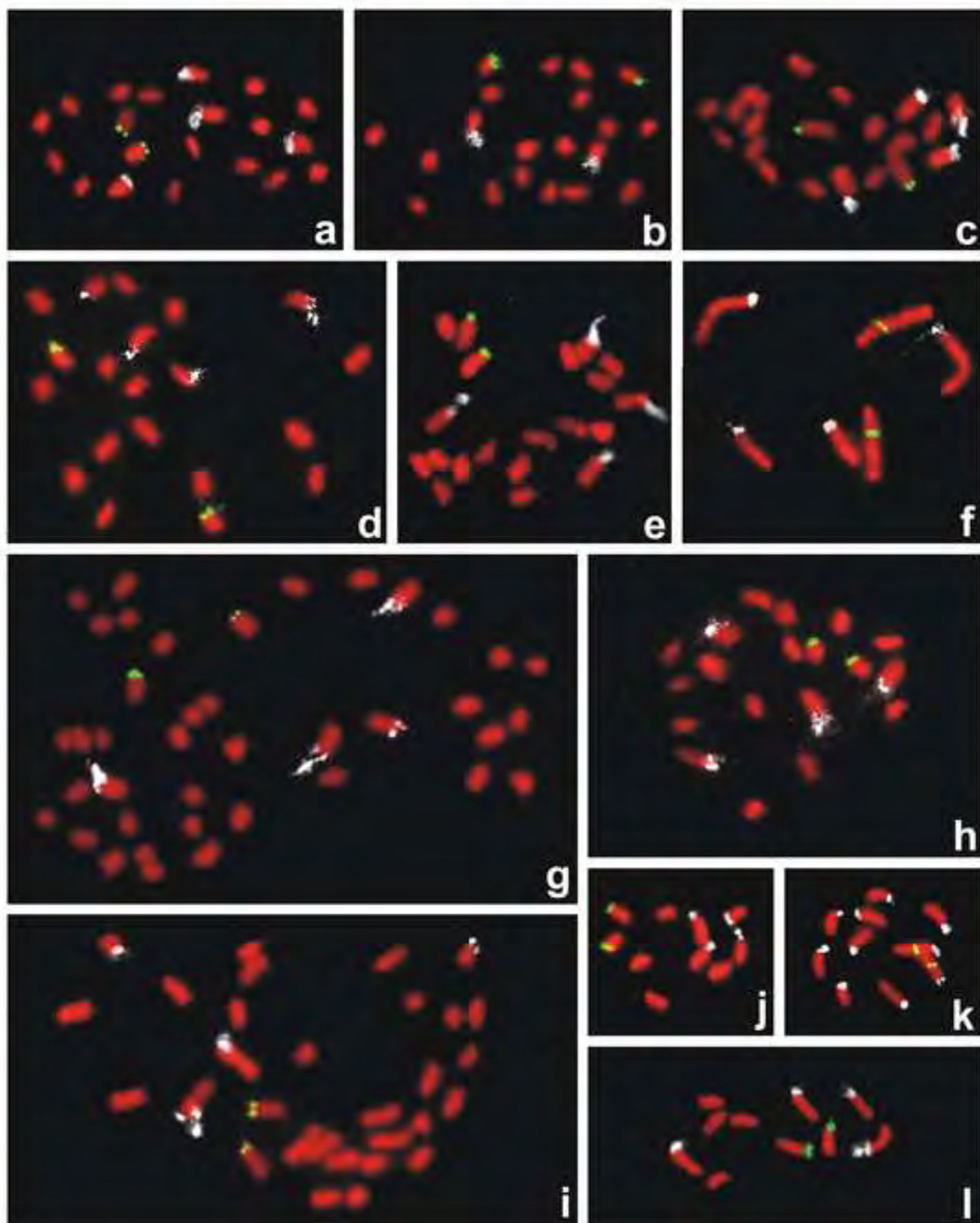
6 **Figure 1.** Metaphases of *Eleocharis* species double-probed with 45S rDNA (white) and  
 7 5S rDNA (greenish-yellow) probes. a) *E. contracta* ( $2n = 20$ ) showing four  
 8 hybridization signals of 45S rDNA and two of 5S. b) *E. nana* ( $2n = 20$ ) with two signals  
 9 of each 45S and 5S rDNA. c) *E. niederleinii* ( $2n = 20$ ) with four 45S rDNA signals and  
 10 two for 5S. d) *E. loefgreniana* ( $2n = 20$ ). e) *E. viridans* ( $2n = 20$ ). f) *E. subarticulata* ( $2n$   
 11  $= 6$ ). g) *E. montana* ( $2n = 40$ ). h) *E. minima* ( $2n = 20$ ). i) *E. filiculmis* ( $2n = 30$ ). j) *E.*  
 12 *capillacea* ( $2n = 10$ ). d, e, g, h, i, exhibited four 45S rDNA and two 5S rDNA signals in  
 13 terminal positions. f) *E. subarticulata*, exhibited 5S signal in interstitial positions. k) *E.*  
 14 *flavescens* ( $2n = 10$ ) with ten 45S rDNA signals and two interstitial 5S signals. l) *E.*  
 15 *maculosa* ( $2n = 10$ ) with four 45S rDNA and two 5S. Bar represents 10  $\mu\text{m}$ .

16

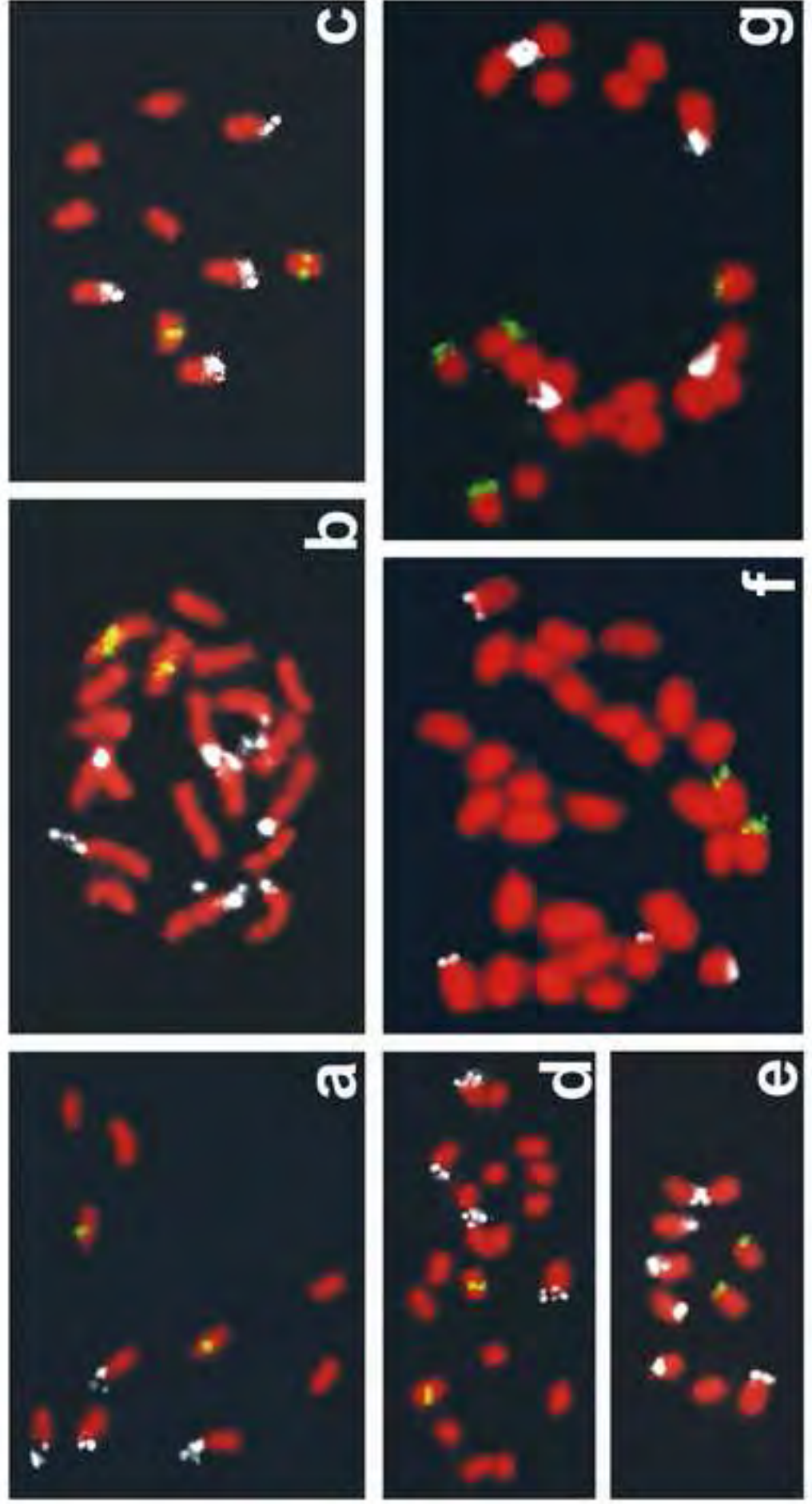
17 **Figure 2.** Metaphases of *Eleocharis* species double-probed with 45S rDNA (white) and  
 18 5S rDNA (greenish-yellow) probes. a) *E. sellowiana* ( $2n = 10$ ) with four 45S rDNA  
 19 signals and two interstitial 5S signals. b) *E. sellowiana* ( $2n = 20$ ) with eight 45S rDNA  
 20 signals and two interstitial 5S signals. c and d) *E. geniculata* ( $2n = 10$  and 20) both with  
 21 four 45S rDNA signals and two interstitial 5S signals, respectively. e) *Eleocharis* sp.  
 22 ( $2n = 10$ ) showing six hybridization signals of 45S rDNA and two of 5S. f) *E. debilis*  
 23 ( $2n = 30$ ) with four 45S rDNA and two 5S. g) *E. bonariensis* ( $2n = 20$ ) with four 45S  
 24 rDNA and four 5S. Bar represents 10  $\mu\text{m}$ .

25

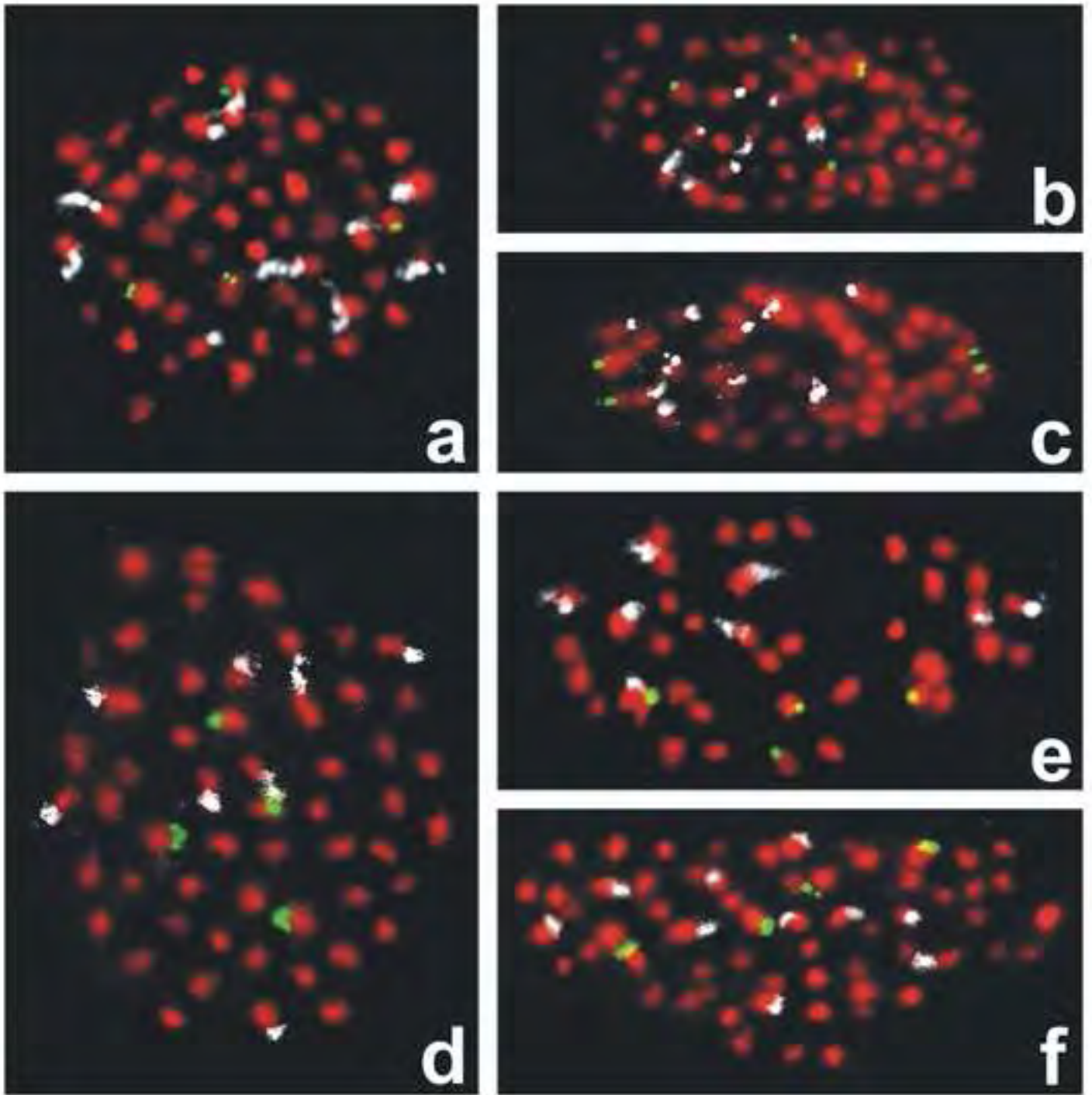
1 **Figure 3.** Metaphases of subgenus *Limnochloa* species double-probed with 45S rDNA  
2 (white) and 5S rDNA (greenish-yellow) probes. a) *E. plicarhachis* ( $2n = 54$ ) showing  
3 ten hybridization signals of 45S rDNA and four of 5S. b) *E. obtusetrigona* ( $2n = 52$ )  
4 with eight hybridization signals of 45S rDNA and four of 5S. c) *E. liesneri* ( $2n = 50$ )  
5 with ten hybridization signals of 45S rDNA and four of 5S. d and e) *E. acutangula* ( $2n$   
6 = 54) and *E. interstincta* ( $2n = 40$ ) both with eight hybridization signals of 45S rDNA  
7 and four of 5S. f) *E. laeviglumis* ( $2n = 60$ ) with ten 45S rDNA and four 5S. Bar  
8 represents 10  $\mu\text{m}$ .



colour figure  
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**Table 1.** Amount and distribution of rDNA hybridization sites in 23 species of *Eleocharis* (Cyperaceae). Species are grouped according the classification of González-Elizondo and Peterson (1997).

Species	2n	45S sites number	CP	5S sites number	CP	Coordinates, localities and voucher numbers
<b>Subgenus <i>Eleocharis</i></b>						
<i>E. contracta</i> Maury	20	4	T	2	T	25°17'48"S 49°54'42"W, Tibagi, PR. 152096
<i>E. nana</i> Kunth	20	2	T	2	T	25°38'37"S 51°40'01"W, Pinhão, PR. 158383
<i>E. loefgreniana</i> Boeck.	20	4	T	2	T	19°13'20"S 43°33'32"W, Serra do Cipó, MG. 152659
<i>E. niederleinii</i> Boeck.	20	4	T	2	T	25°38'37"S 51°40'01"W, Pinhão, PR. 158380
<i>E. minima</i> Kunth	20	4	T	2	T	19°04'13"S 43°24'58"W, Conc. do Mato Dentro, MG. 133
<i>E. viridans</i> Kük ex Osten	20	4	T	2	T	30°06'18"S 50°44'33"W, Viamão, RS. 153094
<i>E. filiculmis</i> Kunth	30	4	T	2	T	20°34'51"S 51°36'36"W, Vestia, MS. 151969
<i>E. montana</i> (Kunth) Roem. & Schult.	40	4	T	2	T	23°13'73"S 51°13'71"W, Londrina, PR. 151964
<i>E. subarticulata</i> (Nees) Boeckl.	6	4	T	2	I	21°49'42"S 46°29'51"W, Poços de Caldas, MG. 152647
<i>E. maculosa</i> (Vahl) Roem & Schult	10	4	T	2	T	30°12'08"S 50°12'52"W, Balneário Pinhal, RS. 158385
<i>E. capillacea</i> Kunth.	10	4	T	2	T	22°15'54"S 47°55'30"W, Brotas, SP. 152642
<i>E. flavescens</i> (Poir.) Urban	10	10	T	2	I	25°50'53"S 48°32'50"W, Caiobá, PR. 156144
<i>E. sellowiana</i> Kunth.	10	4	T	2	I	25°50'53"S 48°32'50"W, Caiobá, PR. 156140
<i>E. geniculata</i> (L.) Roem & Schult.	20	8	T	2	I	20°34'51"S 51°36'36"W, Vestia, MS. 69
<i>E. debilis</i> Kunth.	10	4	T	2	I	26°16'46"S 51°03'24"W, Porto União, SC. 84
<i>E. bonariensis</i> Nees	20	4	T	2	I	21°45'13"S 48°59'55"W, Ibitinga, SP. 151980
<i>Eleocharis</i> sp.	30	4	T	2	T	25°22'51"S 48°51'50"W, Morretes, PR. 156129
<b>Subgenus <i>Scirpidium</i></b>						
<i>E. bonariensis</i> Nees	20	4	T	4	T	28°45'05"S 50°22'09"W, Cambará do Sul, RS. 229
<i>Eleocharis</i> sp.	10	6	T	2	T	25°39'59"S 51°40'05"W, Pinhão, PR. 193
<b>Subgenus <i>Limnochloa</i></b>						
<i>E. interstincta</i> (Vahl) Roem & Schult.	40	8	T	4	T	08°39'40"S 35°09'57"W, Rio Formoso, PE. 11
<i>E. obtuse-trigona</i> Lindl. & Nees.	52	8	T	4	T	25°36'16"S 51°40'74"W, Pinhão, PR. 354
<i>E. liesneri</i> S. González & Reznicek	50	10	T	4	T	21°34'08"S 45°46'77"W, Paraguaçu, MG. 152648
<i>E. laevigulmis</i> R. Trevis. & Boldrini	60	10	T	4	T	29°54'18"S 50°06'15"W, Imbé, RS. 219
<i>E. acutangula</i> (Roxb.) Schult.	54	8	T	4	T	19°43'39"S 50°13'39"W, Iturama, MG. 151977
<i>E. plicarhachis</i> (Griseb.) Svenson	54	10	T	4	T	19°10'55"S 57°42'47"W, Pantanal, MS. 180

T = terminal and I = interstitial rDNA positions; CP = chromosome positions



Mach 03, 2010

Dear Editor of Genetica

I am submitting the manuscript “**Distribution of 45S and 5S rDNA sites in 23 species of *Eleocharis* species (Cyperaceae)**” of Carlos Roberto Maximiano da Silva and André Luís Laforga Vanzela, to evaluation and possible publication at Genetica.

In this cover letter we cleared the following points:

1. we assured that this article was not submitted for publication in any other journal, book, conference proceedings and printed or digital circulation vehicle;
2. we assured that this study generated only this article and that similar versions were not prepared or submitted to other journals;
3. we also assured that we continued studying this theme and that other studies and articles will be accomplished in continuity to this;
4. we assured that all the consultation sources, as well as financing agency are properly mentioned, and that these do not hurt copyrights;
5. and to conclude, we assured that all of the authors have contributed substantially to the manuscript and approved the final submission.

Best regards,

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**Capítulo 4**

Chromosome reduction in *Eleocharis maculosa*  
(Cyperaceae)

Artigo publicado na revista Cytogenet Genome Research 2008 122:175–180

# Chromosome reduction in *Eleocharis maculosa* (Cyperaceae)

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**Abstract.** Chromosome numbers in Cyperaceae lower than the typical basic number  $x = 5$  have been described for only three species: *Rhynchospora tenuis* ( $n = 2$ ), *Fimbristylis umbellaris* ( $n = 3$ ) and *Eleocharis subarticulata* ( $n = 3$ ). *Eleocharis maculosa* is recorded here as the fourth species of Cyperaceae that has a chromosome number lower than  $2n = 10$ , with  $2n = 8, 7$  and  $6$ . The karyotype differentiation in *E. maculosa* was studied using conventional staining (mi-

toxis and meiosis), FISH with 45S and 5S rDNA and telomere probes. The results allow us to determine which chromosomes of the chromosome race with  $2n = 10$  fused to form the remaining reduced numbers, as well as to understand how the symploidy and translocation mechanisms were important in karyotype differentiation and the formation of chromosome races in *Eleocharis*.

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Some plant groups exhibit seemingly conserved karyotypes, with chromosome number, shape and size relatively constant when these are studied by conventional cytogenetics. A good example is the relative similarity among karyotypes of different species of *Lobelia*, Campanulaceae (Ruas et al., 2001), and *Solanum*, Solanaceae (Chiarini and Bernardello, 2006). However, in the family Cyperaceae, intra- and interspecific karyotype variations associated with chromosome number and size are very common. The occurrence of chromosomes with diffuse kinetochore (holocentric chromosomes) is well known in Cyperaceae. When these chromosomes are broken (agmatoploidy) or fused (symploidy) (Luceño and Guerra, 1996), they continue to be viable in the subsequent cell divisions generating different numbers for the same species. This has been demonstrated in experiments with X and  $\gamma$  irradiation of the chromosomes

of *Eleocharis palustris* (L.) Roemer and Schultes (Håkanson, 1954) and *Rhynchospora pubera* (Vahl) Boeck (Vanzela and Colaço, 2002).

Structural rearrangements that culminate in a decrease in chromosome number have been described in different plant groups (Jones, 1998). However, few studies have demonstrated in detail the mechanisms by which such events occur. Among these few cases, we can mention the study of Ikeda (1987) who showed that  $n = 2$  in *Haplopappus gracilis* (Nutt.) A. Gray (Asteraceae) originated by chromosome tandem fusion. Similarly, Lysak et al. (2006) showed a study of chromosome painting where a series of peri- (and para-) centric inversion, followed by reciprocal translocations and meiotic loss of the small product, caused the chromosome number reduction in *A. thaliana* and other Brassicaceae species. In Cyperaceae, numeric reduction below the more common basic number ( $x = 5$ ) in the family has been reported for three species: *Fimbristylis umbellaris* (L.) Vahl with  $n = 3$  (Rath and Patnaik, 1981), *Rhynchospora tenuis* Link with two chromosome races,  $n = 2$  and  $4$  (Vanzela et al., 1996) and *Eleocharis subarticulata* (Nees) Boeck with  $n = 3$  (da Silva et al., 2005). However, only the last two studies showed meiotic configurations compatible with reduction in chromosome number that originated by symploidy and multiple translocations, respectively.

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Request reprints from André Luís Laforga Vanzela  
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**Table 1.** Localities of the analyzed populations of *Eleocharis maculosa* and karyotype features. 2n = diploid number, DSL = diploid set length, FUEL = voucher number, and GPS = global positioning system, ICN = herbarium of the Universidade Federal do Rio Grande do Sul, Brazil.

2n	Natural location in Brazil Cities, States (GPS)	DSL	FUEL
10	Matos Costa, Santa Catarina (W 51°07'52'' – S 26°27'68'') Lebon Regis, Santa Catarina (W 50°21'51'' – S 26°49'67'') Viamão, Rio Grande do Sul (W 50°44'33'' – S 30°06'18'') Balneário Pinhal, Rio Grande do Sul (W 50°12'52'' – S 30°12'08'') São Luis do Purunã, Paraná (W 49°52'93'' – S 25°28'74'') Palmeira (BR 277), Paraná (W 50°09'49'' – S 25°24'49'')	20.96 µm	C.R.M. Silva & A.L.L. Vanzela 85a (ICN) C.R.M. Silva & A.L.L. Vanzela 95 (ICN) C.R.M. Silva & A.L.L. Vanzela 184 (ICN) C.R.M. Silva & A.L.L. Vanzela 216 (ICN) C.R.M. Silva & A.L.L. Vanzela 100 (ICN) C.R.M. Silva & A.L.L. Vanzela 71 (ICN)
8	Palmeira (BR 277), Paraná (W 50°09'49'' – S 25°24'49'') Iporanga, São Paulo (W 48°43'70'' – S 24°31'92'')	20.95 µm	C.R.M. Silva & A.L.L. Vanzela 71a (ICN) C.R.M. Silva & A.L.L. Vanzela 76 (ICN)
7	Bom Sucesso de Itararé, São Paulo (W 49°07'95'' – S 24°11'47'')	20.30 µm	Gaeta M.L. & A.L.L. Vanzela 244 (ICN)
6	Curitiba, Paraná (W 49°16'40'' – S 25°23'04'') Serra do Cipó, Minas Gerais (W 43°33'31'' – S 19°20'28'') Conceição do Mato Dentro, Minas Gerais (W 43°25'03'' – S 19°02'03'') Bom Sucesso de Itararé, São Paulo (W 49°07'95'' – S 24°11'47'')	20.46 µm	C.R.M. Silva 72 (ICN) C.R.M. Silva & A.L.L. Vanzela 142 (ICN) C.R.M. Silva & A.L.L. Vanzela 139 (ICN) Gaeta M.L. & A.L.L. Vanzela 245 (ICN)

About 60 of the more than 200 species of *Eleocharis* are found in Brazil (Trevisan and Boldrini, 2008). *Eleocharis maculosa* (Vahl) Roem. & Schult. occurs from Central America to the south of Brazil (Svenson, 1929; Trevisan and Boldrini, 2008). Chromosome counts in this species indicate the existence of four different chromosome numbers (2n = 10, 8, 7 and 6), 2n = 10 being initially described by da Silva et al. (2008). In order to understand this numeric variation, samples of 11 populations of *E. maculosa* were studied using different cytogenetic tools. The discussion shows how the findings lead to a better understanding of the evolutionary role of the symploidy in the process of karyotype differentiation in the formation of chromosome races in *E. maculosa*.

## Material and methods

Representatives of *Eleocharis maculosa* were collected from eleven flooded regions in five Brazilian states (Table 1). Three to five samples of each population were cultivated in pots at the Laboratório de Biodiversidade e Restauração de Ecossistemas (LABRE) of the Universidade Estadual de Londrina (UEL) to produce new roots and anthers. Vouchers were deposited in the herbarium of the Universidade Federal do Rio Grande do Sul (ICN). For the study of somatic chromosomes, roots were pretreated with 2 mM 8-hydroxyquinoline for 24 h and fixed in absolute ethanol:glacial acetic acid (3:1, v:v) for 12 h and kept at -20 °C until used. Root tips were digested for 3 h in a mixture of 2% (w/v) cellulase and 20% (v/v) pectinase, further hydrolyzed in 1 N HCl at 60 °C for 11 min, dissected in a drop of 45% 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 measured from ten different metaphases of each population, considering each chromosome number.

For meiotic study, spikelets were dissected and the anthers immediately fixed in absolute ethanol:glacial acetic acid (3:1, v:v) for 12 h, and kept at -20 °C until used. Anthers were hydrolyzed in 1 N HCl at 60 °C for 5 min and squashed in a drop of 45% acetic acid. The cover-slips 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 Heslop-Harrison et al. (1991) and Cuadrado and Jouve (1994), with

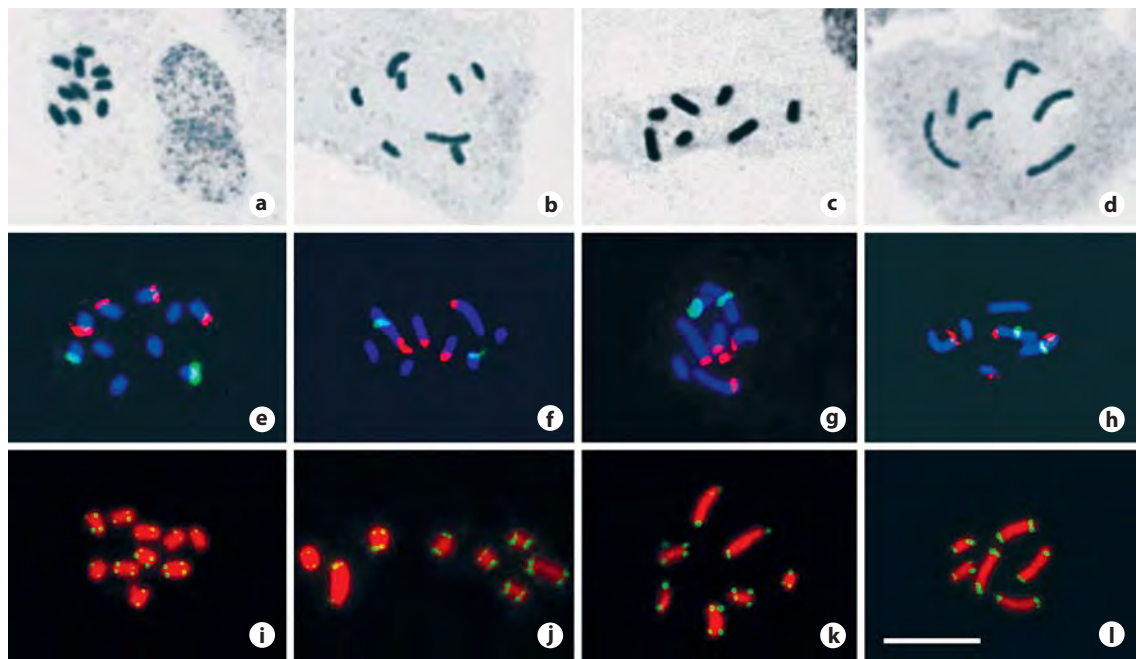
modifications. Slides were prepared with root tips pretreated as described above. Root tips were softened in 2% cellulase plus 20% pectinase at 37 °C for 3 h and squashed in a drop of 45% acetic acid, without acid hydrolysis. The 45S rDNA probe from *Triticum aestivum* and the (TTTAGGG)<sub>n</sub> telomeric probe from *Arabidopsis thaliana* (Richards and Ausubel, 1988) were labeled with biotin-14-dATP and the 5S rDNA probe from *Rhynchospora pubera* (Angeles Cuadrado, unpublished) was labeled with digoxigenin-11-dUTP, both by nick translation. The probes of rDNA were simultaneously utilized for FISH and the telomeric probe was separately hybridized. For FISH, a 34 µl mixture was used composed of 100% formamide (15 µl), 50% polyethylene glycol (6 µl), 20× SSC (3 µl), 100 ng of calf thymus DNA (1 µl), 10% SDS (1 µl), and 200 ng of probes (4 µl each). The material was 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, 20% formamide in 0.1× SSC, 0.1× SSC and 4× SSC/0.2% Tween 20, all at 42 °C. The probes were simultaneously detected with avidin-FITC conjugate and anti-dig-rhodamine conjugate. The post-detection washes were performed in 6× SSC and 4× SSC/0.2% Tween 20, all at room temperature. Slides were mounted with 25 µl of antifade, composed of glycerol (90%), 1,4-diaza-bicyclo(2,2,2)-octane (2.3%), 20 mM TrisHCl, pH 8.0 (2%), 2.5 mM MgCl<sub>2</sub> (4%) and distilled water (1.7%), plus 1 µl of 2 µg ml<sup>-1</sup> 4',6-diamidino-2-phenylindole (DAPI).

All the images were acquired separately without color (black and white) with a Leica DM 4500 B microscope equipped with a DFC 300FX camera and overlapped with blue colors for DAPI, green-yellowish for FITC and red for rhodamine, using the Leica IM50 4.0 software. The images were optimized for best contrast and brightness with iGrafx Image software.

## Results and discussion

### *Morphological analysis and conventional karyotype*

All individuals studied are part of a complex identified as *Eleocharis maculosa* s.l. (Svenson, 1929), belonging to the subgenus *Eleocharis*, section *Maculosae* (González-Elizondo and Peterson, 1997). Independent of the locality, individuals exhibited an apex of upper sheath membranous, rugose and hyaline without a mucron, spikelet ovoid to lanceolate, the scales ovate with scarious margins and reddish to brownish sides that become lighter at age; and obovoid, black achenes. The conventional study performed on 13



**Fig. 1.** Conventional staining and FISH with rDNA probes in *Eleocharis maculosa*. (a) Mitotic metaphase of pleisio-morphic chromosome race with  $2n = 10$  (note the chromocentric interphase nuclei). (b–d) Metaphase and prometaphases in the chromosome races with  $2n = 8$  (b),  $2n = 7$  (c) and  $2n = 6$  (d). (e–h) Double FISH with 45S rDNA (red) and 5S rDNA (green) probes, where the chromosome races are (e)  $2n = 10$ , (f)  $2n = 8$ , (g)  $2n = 7$ , and (h)  $2n = 6$ . The chromosomes were counterstained with DAPI (blue). (i–l) FISH with  $(TTTAGGG)_n$  probe (yellowish green), where the chromosome races are (i)  $2n = 10$ , (j)  $2n = 8$ , (k)  $2n = 7$ , and (l)  $2n = 6$ . The chromosomes were counterstained with DAPI (red). Scale bar = 10  $\mu\text{m}$ .

samples of 11 populations of *E. maculosa* showed four chromosome numbers:  $2n = 10, 8, 7$  and  $6$  (Fig. 1a–d). The number  $2n = 10$  was recorded by da Silva et al. (2008), the other ones are described here for the first time. Given that the samples studied exhibited similar morphological features that do not enable the recognition of infraspecific taxa, we believe that the different karyotypes represent ‘chromosome races’ of *Eleocharis maculosa*.

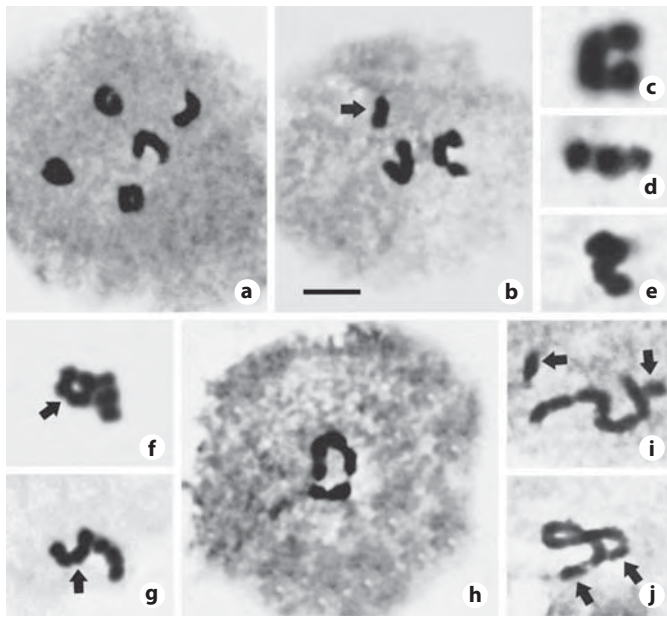
The number  $2n = 10$  was observed in six populations,  $2n = 8$  in two,  $2n = 7$  in one and  $2n = 6$  in four. Most of the populations included individuals with just one chromosome number, except those of Palmeira, Paraná, with  $2n = 10$  and  $8$  and Bom Sucesso de Itararé, São Paulo, with  $2n = 7$  and  $6$  (Table 1). The karyotype with  $2n = 10$  was the most symmetrical, with chromosomes decreasing gradually in size, unlike  $2n = 8$  (two large and six smaller chromosomes),  $2n = 7$  (three large and four smaller) and  $2n = 6$  (four large and two smaller chromosomes) which were bimodals (Fig. 1a–d). In spite of differences found in the sizes of individual chromosomes among the samples, the values of diploid set sizes were similar in all the samples with  $2n = 10, 8, 7$  and  $6$  (Table 1).

Species with more than one chromosome number are common in some genera of Cyperaceae, e.g., *Carex blepharicarpa* Franchet with  $2n = 26–32$  and  $41$  (Hoshino and Okamura, 1994), *C. sociata* Boott with  $2n = 40–44$  (Ohkawa et al., 2000); *Rhynchospora nervosa* (Vahl) Böckeler with

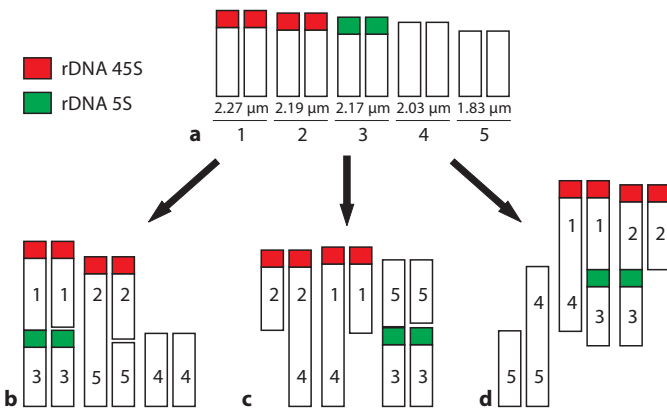
$2n = 20$  and  $30$ , *R. globosa* (H B K.) Roem. & Schult.,  $2n = 24, 37$  and  $48$  (Luceño et al., 1998); *R. confinis* (Nees) C. B. Clarke with  $2n = 10$  and  $20$  (Vanzela et al., 2000), *Eleocharis palustris* subsp. *palustris* (L.) Roem. et Schult.,  $2n = 15$  and  $16$  and *E. palustris* subsp. *vulgaris* Walters,  $2n = 36, 38, 39$  and  $42$  (Strandhede, 1965), and *Eleocharis kamtschatica* (C. A. Mey.) Kom.,  $2n = 41–47$  (Yano and Hoshino, 2006). Several of these studies point out that symploidy and agmatoploidy are responsible for the numeric variations. However, it has not been clearly shown which chromosomes are involved in these rearrangements. The plants studied here showed similar morphological features and size of diploid complement, and so the differences in the chromosome numbers could have originated by fusion of holocentric chromosomes, or symploidy, as proposed by Luceño and Guerra (1996). This event was responsible for the reduction in chromosome number in *Rhynchospora tenuis* Link from the probable basic number  $x = 5$  (Vanzela et al., 1996).

#### FISH and meiotic analysis

The samples studied here showed the same number of rDNA hybridization signals, being always four signals of 45S rDNA and two signals of 5S rDNA. The karyotypes with  $2n = 10$  exhibited only terminal signals, while in the karyotypes with  $2n = 8, 7$  and  $6$ , 45S rDNA signals were always terminal (red color) and 5S rDNA signals were terminal and interstitial (green color) (Fig. 1e–h). Meiotic and FISH anal-



**Fig. 2.** Meiosis in *Eleocharis maculosa*. (a) Metaphase I with five bivalents in the chromosome race with  $2n = 10$ . (b) Chromosome race with  $2n = 8$  showing one bivalent (arrow) and two heteromorphic trivalents in metaphase I. (c–e) Trivalents of chromosome race with  $2n = 8$  in a larger magnification. The smaller chromosomes on the same side of the larger (c), the smaller ones at both ends (d) and one small on each side of the larger chromosome (e). (f and g) Chromosome race with  $2n = 7$  showing one tetravalent (arrows) and one trivalent in chain. Arrows point out a ring tetravalent (f) and a chain tetravalent (g). (h–j) Chromosome race with  $2n = 6$  showing one hexavalent structure (h) and a linear hexavalent (i and j). The arrows point out two small chromosomes located at the ends in (i) and (j). Scale bar = 5  $\mu\text{m}$ .



**Fig. 3.** Idiograms showing the symploidy pathways to formation of chromosome races in *Eleocharis maculosa*. The chromosome arrangement in the idiogram follows possible meiotic pairing. (a) Chromosome races with  $2n = 10$ . The  $\mu\text{m}$  values correspond to the average of the size of each pair. (b–d) Chromosome race with  $2n = 8$ , 7 and 6, respectively.

yses allowed us to propose the pathways in which the chromosome reduction occurred from one plesiomorphic karyotype ( $2n = 10$ ) with terminal hybridization sites to different karyotypes with interstitial and terminal sites of rDNA. Meiotic analysis performed in six samples with  $2n = 10$  showed for all cases the presence of five bivalents at metaphase I (Fig. 2a), comprising a regular meiosis typical of a plesiomorphic karyotype in the context of *Eleocharis maculosa*. However, the samples with reduced chromosome numbers displayed different configurations in the first phase of meiosis.

*The chromosome race with  $2n = 8$ :* In the two samples with  $2n = 8$ , the formation of one bivalent was observed, composed probably of two smaller chromosomes, and two different heteromorphic trivalents, each one composed of a larger chromosome pairing with two smaller chromosomes (Fig. 2b). The trivalents appeared arranged in at least three different forms: (i) the largest chromosome with two smaller on the same side (Fig. 2c), (ii) linear with the smaller ones at both tips (Fig. 2d), and (iii) a small chromosome on each side of the larger one (Fig. 2e). FISH with rDNA probes revealed that one of the largest chromosomes had two hybridization signals. One of these was terminal (45S rDNA) and the other was interstitial (5S rDNA). According to our chromosome measurements, the largest chromosome can be the result of the fusion of a homolog of pair 1 with a homolog of pair 3 (see Fig. 3). Due to this association, these heterologous chromosomes appear as a heteromorphic trivalent at meiosis (see Fig. 2b). The second largest chromosome showed only one terminal signal with the 45S rDNA probe. This might have been the result of the fusion of a homolog of pair 2 probably with a homolog of pair 5, which is suggested also by the chromosome measurements (see Fig. 3). In this case, this association appears also as a heteromorphic trivalent at meiosis (see Fig. 2b). Three other chromosomes with similar sizes exhibited terminal signals, two with the 45S rDNA and one with the 5S rDNA probe. The other three smallest do not show hybridization signals (Figs. 1f, 3). Finally, the only bivalent may be formed by two chromosomes of pair 4 (Fig. 3).

*The chromosome race with  $2n = 7$ :* FISH analysis of mitotic chromosomes with rDNA probes showed that the three largest chromosomes exhibited only one hybridization signal each. Two chromosomes showed terminal signals with the 45S rDNA probe and the other chromosome showed one interstitial signal with the 5S rDNA probe. Three of the four other smaller chromosomes displayed a terminal signal, where two were hybridized by the 45S rDNA probe and one by the 5S rDNA probe (Fig. 1g). According to our chromosome measurements, the largest chromosome is the result of the fusion of a homolog of pair 1 with a homolog of pair 4. The second largest chromosome may be the result of the fusion of a homolog of pair 2 probably with a homolog of pair 5. The remaining smaller chromosomes, exhibited terminal signals, two with 45S rDNA and one with 5S rDNA, except for one without signals (Fig. 3c). Mei-



otic analysis done on this chromosome race always showed one tetravalent and one trivalent, both heteromorphic. The tetravalent appears forming a ring structure or in a chain, and the trivalent always appears in a chain (Fig. 2f, g). The tetravalent configuration can be a result of the pairing among fused chromosomes 1/4 and 2/4, besides the homologous chromosomes 1 and 2, which are not fused. The trivalent was composed of fused chromosome 3/5 associated with its non-fused homologs, as suggested also by chromosome measurements (Fig. 3). However, we cannot reject the possibility of translocations, because ring structures were found, as observed in *E. subarticulata* (da Silva et al., 2005).

*The chromosome race with 2n = 6:* Meiotic analysis of the samples with  $2n = 6$  showed that all chromosomes appeared as a single multivalent structure. In most cases the chromosomes formed a hexavalent structure, but not in a ring form (Fig. 2h–j). Da Silva et al. (2005) observed one hexavalent ring in *Eleocharis subarticulata*, however, it originated by multiple translocations. In the chromosome race with  $2n = 6$  we found a multivalent association with two chromosomes of small size located at the extremes, but not physically associated with each other (Fig. 2i, j). FISH with rDNA probes showed that in mitotic metaphases the first and second largest chromosomes, which differed in size, each exhibited two hybridization signals.

These were terminal with the 45S rDNA probe and interstitial with the 5S rDNA probe (Fig. 1h). According to our analysis, the two homologs of pair 3, containing the 5S rDNA sites, were fused with two different heterologous chromosomes, one of pair 1 and the other of pair 2 and both containing 45S rDNA sites (Figs. 1h, 3d). Continuing the reasoning for the explanation of the formation of the hexavalent structure, the two homologs of pair 4, without rDNA sites, were also fused with two different heterologous chromosomes, one from pair 1 (containing 45S rDNA site) and the other from pair 5 without rDNA sites (Fig. 3d). Thus, the two small chromosomes located at the ends of the hexavalent structure may correspond to the chromosomes of pairs 2 and 5 that were not fused (Fig. 3d).

The use of conventional and molecular techniques allows us to identify most of the chromosomes that participated in the fusion processes, as well as the different combinations of the chromosomes giving rise to each reduced karyotype. This combination of different procedures has been useful in identifying chromosomes in other plant groups that exhibit rearrangements, e.g., *Arabidopsis* (Lysak et al., 2006) and *Brachyscome* (Adachi et al., 1997). FISH with (TTTAGGG)<sub>n</sub> probes was used here to verify if there was the maintenance of ectopic segments after process of chromosome reduction in *E. maculosa*. The absence of ectopic signals in the samples with  $2n = 10, 8, 7$  and  $6$  (Fig. 1i–l) could indicate that the chromosome races appeared from translocations with terminal breakpoints and subsequent loss of small products (with telomeres), as found in *Arabidopsis* (Lysak et al., 2006). However, breaks in chromosomes with diffuse kinetochores could originate mini or micro-chromosomes with kinetic activity and be viable in

cell division (see Håkanson, 1954 and Vanzela and Colaço, 2002). But, these little chromosomes were not found in our study. We suggested that the fusion process happened due to symploidy of holocentrics, as described in *Rhynchospora tenuis* with  $2n = 4$  and  $8$  (Vanzela et al., 2003).

Interstitial telomeric sites have been shown in *Haplopappus gracilis* with  $2n = 4$  (Hanmoto et al., 2007) and in *Eleocharis subarticulata* with  $2n = 6$  (da Silva et al., 2005) due to chromosome fusion and multiple translocation processes, respectively. According to Schubert (2007) telomeric sequences may occupy interstitial positions because of translocation or inversion, and can be considered to be hot spots of chromosome breakage. Chromosome fusions without the maintenance of telomeric ectopic sites have also taken place in other plant groups with monocentric chromosomes (Cox et al., 1993; Fuchs et al., 1995). The ‘minimum-interaction hypothesis’ of Imai et al. (1986), elaborated in monocentric systems, states that karyotype evolution has been in large part shaped by selection to reduce the occurrence of such fitness-reducing spontaneous chromosomal mutations as reciprocal translocations. Thus, we suggested symploidy as the principal chromosome reduction mechanism in *E. maculosa*. However, we cannot discard the translocation in the chromosome races formation, since a ring configuration was found in meiosis of the race with  $2n = 7$ .

Jones (1998) mentions that most of the suspected cases of chromosome fusion and fission are records of karyotype comparisons of small numbers of individuals. Most of the records involve groups with monocentric chromosomes, whose karyotype evolution tends to increase chromosome numbers, mainly by polyploidy, where reduction in chromosome number is rarely considered (Schubert, 2007). In the context of holocentric chromosomes, the pathways of karyotype evolution also seem to be associated with increased chromosome numbers, due to agmatoploidy and polyploidy. In *Carex*, the most studied genus of Cyperaceae, a decrease in chromosome number is not common (Hipp et al., 2008). Thus, considering that most of the chromosome numbers recorded for *Eleocharis* are equal to or larger than  $2n = 10$ , with  $x = 5$  as the probable basic number, the chromosome races found in *E. maculosa* can be an apomorphic feature. In conclusion, our findings reinforce the suggestion of Guerra (2008) that descending disploidy, reaching very small chromosome numbers, is more frequent in organisms with holokinetic chromosomes.

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**Capítulo 5**

Análises citogenéticas e moleculares revelam um  
complexo de híbridos e poliploides em *Eleocharis* série  
*Tenuissimae* (Cyperaceae)

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1 **Análises citogenéticas e moleculares revelam um complexo de híbridos e**  
2 **poliploides em *Eleocharis* série *Tenuissimae* (Cyperaceae)**

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17

18 **Resumo**

19 O gênero *Eleocharis* R. Br. possui características morfológicas bastante simples,  
20 quando comparado com as demais Cyperaceae. Isto tem ocasionado problemas de  
21 identificação e classificação, os quais não têm sido resolvidos com o auxílio de  
22 ferramentas moleculares. Um dos grupos mais complexos do gênero é a série  
23 *Tenuissimae* (C. B. Clarke) Svenson, de origem parafilética. Esta série exibe a maior  
24 diversidade morfológica no gênero, incluindo complexos de espécies intimamente  
25 relacionadas, como o complexo formado por *Eleocharis viridans* e *E. niederleinii*. Para  
26 compreender a organização e evolução deste complexo, foram estudadas 39 amostras  
27 coletadas em diversas regiões do Brasil. As análises revelaram uma série cromossômica  
28 para ambas as espécies, as quais exibiram  $2n = 20, 29, 30, 31, 35, 40, 41$  e  $42$ . Em  
29 alguns cariótipos foram encontrados de um a quatro cromossomos maiores, contendo ou

1 não sítios de DNAr 45S. As poucas amostras que produziram flores férteis apresentaram  
2 meiose regular, apenas com bivalentes. Os dados moleculares indicam que as  
3 populações podem estar isoladas e a GISH revelou que duas destas populações têm  
4 origem híbrida. Esses híbridos possuem reprodução exclusivamente vegetativa, o que  
5 indica a ocorrência de isolamento ativo.

6

7

8 **Palavras-chave:** Aloploidia, DNAr 45S, *Eleocharis*, GISH, hibridação.

## 1 **Introdução**

2 O gênero *Eleocharis* R. Br. possui características morfológicas bastante  
3 reduzidas, quando comparado com as demais Cyperaceae (Trevisan e Boldrini 2008),  
4 associadas a uma grande variação morfológica intraespecífica (Simpson 1988). Isto tem  
5 ocasionado problemas de identificação e classificação, os quais vêm se mantendo  
6 mesmo com o auxílio de ferramentas moleculares (Roalson e Friar 2000, Yano *et al.*  
7 2004 e Roalson *et al. in press*). González-Elizondo e Peterson (1997) dividiram o  
8 gênero *Eleocharis* em quatro subgêneros, seis seções, oito séries e sete subséries. Dentre  
9 estes, encontra-se a série *Tenuissimae* (C. B. Clarke) Svenson, de origem para e  
10 polifilética (Roalson e Friar 2000). Esta série exibe a maior diversidade morfológica e  
11 distribuição no gênero, incluindo complexos de espécies intimamente relacionadas,  
12 especialmente na América do Sul, indicando especiação ativa (González-Elizondo e  
13 Tena-Flores 2000).

14 Estudos citogenéticos em *Eleocharis*, assim como nos outros representantes de  
15 Cyperaceae, mostram que há uma grande variação cromossômica numérica, desde  $2n =$   
16 6 em *E. subarticulata* (Da Silva *et al.* 2005) a  $2n = 200$  em *E. dulcis* (Roalson 2008).  
17 Esta variação está associada à condição holocêntrica dos cromossomos, devido a  
18 eventos de agmatoploidia, simploidia, translocações e poliploidia (Luceño *et al.* 1998,  
19 Da Silva *et al.* 2005, 2008a, Hipp *et al.* 2009). Há evidências de que em algumas  
20 espécies, esses eventos podem levar a algum tipo de isolamento, como em  
21 *Rhynchospora tenuis*, já que intermediários entre as raças cromossômicas simpátricas  
22 com  $2n = 4$  e 8 nunca foram encontrados (Vanzela *et al.* 1996). Similarmente, para *E.*  
23 *maculosa* foram encontradas populações com  $2n = 10, 8, 7$  e 6 (Da Silva *et al.* 2008b),  
24 cujos cariótipos surgiram a partir de  $2n = 10$ , sem indícios de cruzamentos entre essas  
25 raças cromossômicas.

1 Estudos prévios realizados em representantes de *E. viridans* Boeck e *E.*  
2 *niederleinii* Kük. Ex Osten da série *Tenuissimae*, revelaram diferentes números  
3 cromossômicos e organização cariotípica, além de uma clara sobreposição de caracteres  
4 diagnósticos. De acordo com Svenson (1939), *Eleocharis viridans* e *E. niederleinii*  
5 podem ser sinônimas, contudo, diferentes taxonomistas consideram *E. viridans* como  
6 uma espécie bem circunscrita. Essas espécies apresentam colmos quadrangulares,  
7 variando de 0,2 -1,1 µm de largura, comportamento prolífero e raras vezes formam  
8 espiguetas viáveis, levando a uma escassez de caracteres diagnósticos e erros na  
9 identificação. Este problema pode ser bem exemplificado em Rambo (1959) na  
10 separação entre *E. viridans* e *E. filiculmis*, em Trevisan e Boldrini (2008) entre *E.*  
11 *viridans* e *E. dunensis*, bem como na determinação errônea de *E. niederleinii* registrada  
12 em vários herbários brasileiros (Alves *et al.* 2010). Para entender a relação entre  
13 *Eleocharis viridans* e *E. niederleinii*, neste estudo foram empregadas ferramentas  
14 citogenéticas, como coloração convencional, para análises meióticas e mitóticas, FISH  
15 com sonda de DNAr 45S e GISH e moleculares como, o RAPD, em 39 amostras dessas  
16 duas entidades taxonômicas, coletadas em 18 localidades das regiões Sul e Sudeste do  
17 Brasil.

18

## 19 **Materiais e Métodos**

20

21 As análises morfológicas dividiram as 39 amostras coletadas, em duas espécies,  
22 19 foram determinadas como *E. niederleinii* e 20 como *E. viridans* (Tabela 1). Estas  
23 amostras foram mantidas em casa de vegetação do LABRE (Laboratório de  
24 Biodiversidade e Restauração de Ecossistemas), do Centro de Ciências Biológicas da

1 Universidade Estadual de Londrina. As exsicatas foram depositadas no herbário da  
2 Universidade Federal do Rio Grande Sul (ICN).

3

#### 4 *Citogenética convencional*

5 Pontas de raízes foram pré-tratadas com 2 mM 8-hidroxiquinoleína por 24 h e  
6 fixadas em etanol absoluto:ácido acético glacial (3:1, v:v) por 12 h e mantidas a -20 °C  
7 até o uso. As pontas das raízes foram digeridas por 3 h a 37 °C em uma mistura de 4%  
8 celulase e 40% pectinase (w:v), posteriormente hidrolisadas em HCl 1N a 60 °C por 10  
9 min e lavadas com água destilada. As amostras foram dissecadas e esmagadas em uma  
10 gota de ácido acético a 60% e coradas com Giemsa 2%. O tamanho dos cromossomos e  
11 o comprimento total do cariótipo foram medidos em dez diferentes metáfases de cada  
12 população com o programa MicroMeasure 3.3.

13 Para o estudo das meioses, anteras foram fixadas em etanol absoluto:ácido  
14 acético glacial (3:1, v:v), por 12 h, e mantidas a -20 °C até o uso. As anteras foram  
15 hidrolisadas em HCl 1N a 60 °C, por 9 min, e lavadas em água destilada.  
16 Posteriormente, as amostras foram dissecadas em uma gota de ácido acético a 60% e  
17 coradas com Giemsa 2%.

18

#### 19 *Análises cito-moleculares*

20 A hibridação *in situ* fluorescente (FISH) foi feita de acordo com Heslop-  
21 Harrison *et al.* (1991), com modificações. A sonda de DNAr 45S obtida de *Triticum*  
22 *aestivum* (pTa71) foi marcada com biotina-14-dATP por *nick translation*. Entre 100 e  
23 200 ng (4 µL) da sonda foi adicionada a uma solução composta por formamida 100%  
24 (15 µL), polietilenoglicol 50% (6 µL), 20× SSC (3 µL), 100 ng de DNA de timo de  
25 bezerro (1 µL), SDS 10% (1 µL) e H<sub>2</sub>O (4 µL). A sonda foi desnaturada a 70 °C por 10



1 min e a hibridação feita a 37 °C por até 24 h. Os banhos pós-hibridação foram feitos  
2 com 6× SSC e 4 SSC/0,2% Tween 20, à temperatura ambiente. A sonda foi detectada  
3 com avidina-FITC e os banhos pós detecção foram com 4× SSC/0,2% Tween 20 a  
4 temperatura ambiente. Os cromossomos foram contra-corados com DAPI e as lâminas  
5 foram montadas com 25 µL de antifade, composto por glicerol (90%), 1,4-diaza-  
6 bicyclo(2,2,2)-octane (2.3%), 20 mM TrisHCl, pH 8.0 (2%) e água destilada.

7        Para a hibridação genômica *in situ* (GISH) foi utilizado o mesmo protocolo da  
8 FISH, exceto pelas sondas utilizadas na hibridação, as quais foram obtidas do DNA  
9 genômico extraído das amostras com  $2n = 20$  de *E. viridans* (amostra 186) e *E.*  
10 *niederleinii* (amostra 44). Amostras de DNA genômico das duas espécies foram  
11 fragmentadas por choque de temperatura, avaliadas em gel de eletroforese a 1% e em  
12 seguida marcadas com biotina-14-dATP por *nick translation*. As hibridações *in situ*  
13 foram feitas baseadas nos resultados das análises de RAPD e do coeficiente de  
14 similaridade entre os genomas de cada amostra.

15        Todas as imagens, convencionais, FISH e GISH, foram capturadas em tons de  
16 cinza em um fotomicroscópio Leica DM 4500 B equipado com câmera DFC 300FX. As  
17 sobreposições e colorações das imagens foram feitas no programa IM50 da Leica e o  
18 controle de brilho e contraste feito com o programa IGrafix.

19

## 20 *Extração de DNA*

21        O DNA genômico de 39 indivíduos das duas espécies foram extraídos de acordo  
22 com o método CTAB (Brasileiro e Carneiro 1998), onde cerca de 3 a 4 colmos jovens  
23 foram transferidos para um almofariz contendo nitrogênio líquido e macerados até a  
24 obtenção de um pó fino. Cerca de 2 mL de tampão CTAB (Tris-HCl 1M pH 8, NaCl  
25 5M, EDTA 0,5M pH 8, CTAB, βmercaptoetanol e PVP 40) foi adicionado ao pó e a

1 solução foi transferida para um microtubo. A solução foi incubada em banho-maria a 65  
2 °C, por 50 min, tratada com Proteinase K (100 mg/mL) a 65 °C, por mais 30 min. Após  
3 este período foi adicionado o mesmo volume de fenol:clorofórmio (1:1; v:v) e a solução  
4 foi centrifugada a 10.000 rpm, por 10 min. Após a centrifugação, a fase superior foi  
5 transferida para outro tubo e tratada com RNase (100 mg/mL) a 37 °C por 30 min.  
6 Após este período duas lavagens com clorofórmio:álcool isoamílico (24:1; v:v) foram  
7 realizadas. A fase superior foi transferida para outro tubo e a ela foi adicionado 2,5  
8 volumes de etanol absoluto para formar um precipitado. Este foi dissolvido em 50-100  
9 µL de água destilada. As amostras foram quantificadas em um fluorômetro DyNA  
10 Quant200 (Hoefer-Pharmacia).

11

### 12 *Reação de RAPD*

13 As reações de RAPD foram realizadas em apenas 39 amostras. Para estas  
14 reações, foram testados 36 *primers*, dos quais oito do kit OPA (Operon Technologies –  
15 Alameda, CA) foram selecionados. A reação de amplificação foi feita em um total de 15  
16 µL, contendo 1 mM dNTP, 0,2 mM de primer, 2,5 mM de MgCl<sub>2</sub> e uma unidade de  
17 enzima (DNA polimerase), 1× o Tampão de reação e 15 a 25 ng de DNA e H<sub>2</sub>O Qsp.  
18 As amplificações foram feitas em um termociclador MJ Research PTC-100 com a  
19 seguinte programação: 92 °C, por 4 min, seguido por 40 ciclos, sendo que cada ciclo  
20 consiste em 40 seg, a 92 °C, 1 min e 30 seg, a 40 °C e 2 min a 72 °C, e uma extensão  
21 final de 5 min, a 72 °C após o último ciclo. Ao término da reação de amplificação, foi  
22 adicionado 4 µL de tampão da amostra (glicerol e azul de bromofenol) e a solução  
23 aplicada em um gel de agarose 1,4%, submerso em tampão de corrida TEB 0,5× (Tris  
24 0,045 M, EDTA 0,5 mM e Ácido Bórico 0,045 M, pH 8,3). A eletroforese aconteceu a 3

1 W/cm, o gel foi corado com brometo de etídio por 5 minutos, exposto em  
2 transiluminador ultravioleta e a imagem capturada com o programa Alpha Digi Doc RT.

3 Os perfis de RAPD foram determinados por comparação das bandas produzidas  
4 para cada amostra em cada um dos oito *primers* e os dados comparados na forma de  
5 variáveis binárias (1 presença e 0 ausência). Para as análises de similaridade genética  
6 entre os indivíduos dos diferentes pontos foi utilizado o programa NTSYS-PC. Para a  
7 construção do dendograma de similaridade genética foram empregados o coeficiente de  
8 Jaccard (J) e o método de agrupamento UPGMA (*Unwweighted Pair-Group Method*  
9 *with Aritimetal Average*). O programa STRUCTURE 2.2 (Pritchard *et al.* 2000; Falush  
10 *et al.* 2003) foi empregado para determinar, por análise Bayesiana de agrupamento, o  
11 número de entidades (K) geneticamente distintas. Neste caso, foi utilizada a  
12 configuração para marcadores dominantes, conforme estabelecido por Falush *et al.*  
13 (2007). Para ambas as espécies, as simulações foram realizadas com 100.000 repetições  
14 pelo método Monte Carlo para a Cadeia de Markov (MCMC), após um período de  
15 *burning* de 20.000 repetições. Essas simulações foram baseadas no modelo de mistura,  
16 usando a opção de frequências alélicas correlacionadas, sem determinar *a priori* a  
17 origem dos indivíduos. Duas metodologias foram empregadas para a escolha do número  
18 mais provável de populações inferidas a partir do programa STRUCTURE 2.2. Os  
19 resultados foram utilizados para direcionar as reações de hibridação genômica *in situ*.

## 21 **Resultados**

22 Todas as 39 amostras exibiram proliferação, ou seja, na extremidade do colmo,  
23 novos colmos foram produzidos. Contudo, 22 delas também produziram espiguetas. As  
24 amostras foram classificadas em duas espécies, sendo *E. niederleinii* aquelas que  
25 apresentaram colmos de 0,2–0,4 mm de largura, bainha com 1,1–3,5 cm de

1 comprimento, ápice oblíquo e bordo inconspícuo ou com pontos vináceos. Destas, oito  
2 produziram espiguetas, que tiveram  $3-9 \times 1-2$  mm de tamanho, forma lanceolada com  
3 cor castanha, 10-18 flores; gluma inferior estéril, e glumas superiores frágeis e não  
4 imbricadas (Tabela 1). Foram consideradas *E. viridans* as amostras mais robustas com  
5 colmos de 0,3-1mm de largura, bainha com 1,5-8 cm de comprimento e ápice oblíquo.  
6 Das 20 amostras consideradas *E. viridans*, sete produziram espiguetas (Tabela 1), que  
7 tiveram  $6-13 \times 2-3$  mm de tamanho, com forma ovóide e cor castanha a vinácea, com  
8 30-60 flores; gluma inferior estéril, e glumas superiores imbricadas. Estas duas  
9 entidades apresentaram como características sobrepostas a ausência de apêndice  
10 hialino/rugoso e múcron dorsal.

11 As análises citogenéticas mostraram uma grande variação no número  
12 cromossômico de ambas as espécies. As 19 amostras de *E. niederleinii* exibiram  $2n =$   
13 20, 29, 30, 31, 35 e 40 (Figura 1A - I). As 20 amostras de *E. viridans* exibiram  $2n = 20,$   
14 29, 31, 40, 41 e 42 cromossomos (Figura 2 A - H). Várias amostras de ambas as  
15 espécies exibiram cariótipos contendo cromossomos bem maiores que os demais, os  
16 quais variaram de 3.16 a 4.74  $\mu\text{m}$  (Tabela 1). O comprimento total do lote diplóide foi  
17 similar para os cariótipos com o mesmo número cromossômico independente da espécie  
18 (Tabela 1).

19 A análise meiótica mostrou bivalentes apenas nas amostras com  $2n = 20$  de  
20 ambas as espécies (Figura 3A),  $2n = 30$  de *E. niederleinii* (Figura 3B) de  $2n = 42$  para  
21 *E. viridans* (Figura 3C), em todos estes casos aquênios foram produzidos. As amostras  
22 de *E. niederleinii* com  $2n = 30$  coletadas em Sapopema e Porto União (veja a Tabela 1),  
23 produziram poucas inflorescências e apenas 24 meiócitos puderam ser analisados. Estes  
24 exibiram diferentes configurações, com  $1^{\text{IV}} + 2^{\text{III}} + 9^{\text{II}} + 2^{\text{I}}$  (Figura 3D) observados em  
25 11 células,  $1^{\text{IV}} + 5^{\text{III}} + 4^{\text{II}} + 3^{\text{I}}$  em quatro células,  $2^{\text{IV}} + 2^{\text{III}} + 6^{\text{II}} + 3^{\text{I}}$  em três células,  $1^{\text{IV}}$

1  $3^{\text{III}} + 7^{\text{II}} + 3^{\text{I}}, 1^{\text{IV}} + 3^{\text{III}} + 6^{\text{II}} + 5^{\text{I}}$  e  $1^{\text{IV}} + 4^{\text{III}} + 4^{\text{II}} + 6^{\text{I}}$ , encontradas duas vezes para cada  
 2 configuração.

3 Múltiplas configurações também foram encontradas em amostras de *E. viridans*  
 4 com  $2n = 31$  coletadas em Caçador/SC. Dos 15 meiócitos analisados, seis mostraram  $7^{\text{III}}$   
 5  $+ 3^{\text{II}} + 4^{\text{I}}$  (Figura 3E), quatro mostraram  $1^{\text{VI}} + 1^{\text{IV}} + 5^{\text{III}} + 2^{\text{II}} + 2^{\text{I}}$  (Figura 3F), duas  $1^{\text{VI}} +$   
 6  $4^{\text{III}} + 5^{\text{II}} + 3^{\text{I}}$ , duas  $1^{\text{V}} + 5^{\text{III}} + 4^{\text{II}} + 3^{\text{I}}$  e uma exibiu  $1^{\text{V}} + 1^{\text{IV}} + 4^{\text{III}} + 3^{\text{II}} + 4^{\text{I}}$ . Além das  
 7 meioses irregulares, diferentes números cromossômicos foram encontrados na mitose  
 8 polínica. Das 56 células analisadas, 26 apresentaram  $n = 15$ , com dois cromossomos  
 9 muito maiores que os demais (Figura 3 H), 15 com  $n = 17$  (Figura 3I), cinco com  $n =$   
 10 16, quatro com  $n = 15$  e os números  $n = 13, n = 14$  e  $n = 15$ , foram verificados duas  
 11 vezes, e em todas estas células, um cromossomo muito maior que os demais foi  
 12 observado.

13 Para as amostras de *E. viridans* com  $2n = 41$  coletadas em Balneário Pinhal e  
 14 Capivari do Sul, foram analisadas 63 meiócitos. Destes, 38 células exibiram  $1^{\text{III}} + 19^{\text{II}}$   
 15 (Figura 3G) e 25 células apresentaram  $20^{\text{II}} + 1^{\text{I}}$ . Também foram encontrados diferentes  
 16 números cromossômicos na mitose polínica. Das 73 células analisadas, 34 exibiram  $n =$   
 17 22 (Figura 3J), 23 mostraram  $n = 21$  (Figura 3K),  $n = 20$  foi encontrada em 13 células e  
 18 apenas três exibiram  $n = 23$ . Em todas estas células sempre dois cromossomos maiores  
 19 foram observados.

20 A hibridação *in situ* realizada com a sonda de DNAr 45S, mostrou de quatro a  
 21 seis sinais, sempre terminais nas duas espécies. *Eleocharis niederleinii* mostrou quatro  
 22 sinais nas amostras com  $2n = 20, 29$  e  $40$  (Figuras 4A, B e G), e seis sítios nas amostras  
 23 com  $2n = 30, 31, 35$  e  $40$  (Figuras 4C, D, E, H e I). *Eleocharis viridans* mostrou quatro  
 24 sítios nas mostras com  $2n = 20, 29$  e  $41$  (Figuras 5A, D e H), e seis sítios nas amostras

1 com  $2n = 29, 31, 40, 41$  e  $42$  (Figuras 5B, C, E, F e G). Variações nos tamanhos dos  
2 sinais de hibridação foram encontradas em todas as amostras estudadas.

3 O dendrograma gerado pelo método de UPGMA agrupou as amostras em dois  
4 grandes grupos, considerando a similaridade de 80%, contudo, houve uma mistura de  
5 espécies e localidades. O primeiro grupo foi composto por amostras do Paraná (várias  
6 amostras de *E. niederleinii* e apenas um indivíduo de *E. viridans*), Santa Catarina (*E.*  
7 *niederleinii* e *E. viridans*) e Rio Grande do Sul (somente *E. viridans*), e o segundo por  
8 amostras de Minas Gerais (*E. niederleinii*), Rio Grande do Sul (*E. viridans*) e Paraná  
9 (apenas um indivíduo de *E. niederleinii*). As amostras de uma mesma região foram  
10 agrupadas com alto coeficiente de similaridade (Figura 6). O dendrograma indica que  
11 em um mesmo estado as duas espécies podem ser encontradas, porém, em cada ponto  
12 coletado indivíduos de apenas uma espécie foram amostrados, com exceção de  
13 Pinhão/PR. A análise com o *software* Structure apontou a possível existência de sete  
14 grupos bem estruturados, sendo três de *E. niederleinii* e quatro de *E. viridans* (Figura.  
15 6), indicando um possível isolamento para essas amostras.

16 Para a realização da GISH os DNAs genômicos de *E. niederleinii* coletada em  
17 Alexandrita/MG e *E. viridans* coletada em Viamão/RS, ambas com  $2n = 20$ , foram  
18 utilizadas como sonda. A sonda obtida da amostra de *E. niederleinii*, foi hibridada nas  
19 amostras de *E. niederleinii* com  $2n = 40$  coletadas em Pinhão/PR e São Luis do  
20 Purunã/PR,  $2n = 30$  coletada em Tibagi/PR e na amostra com  $2n = 35$  coletada em  
21 Conceição do Mato Dentro/MG. Em nenhum desses casos sinais de hibridação foram  
22 observados. Esta mesma sonda foi hibridada também em uma amostra de *E. viridans*  
23 com  $2n = 41$  de Capivari do Sul/RS, e novamente nenhum sinal de hibridação foi visto.  
24 A mesma sonda foi hibridada na amostra de *E. niederleinii* com  $2n = 40$  coletada em

1 Alexandrita, neste caso sinais de hibridação foram observados em quase todos os  
2 cromossomos (Figura 7A).

3 A sonda de *E. viridans* hibridou nas amostras de *E. viridans* com  $2n = 42$   
4 coletadas em Pinhão/PR e Lapa/SC, na amostra de  $2n = 40$  de Pinhão e na amostra com  
5  $2n = 29$  de Irani/SC, contudo o padrão de hibridação foi diferente entre elas. Na  
6 amostra com  $2n = 42$  de Pinhão, a sonda hibridou na região terminal de 13  
7 cromossomos e em regiões intersticiais em dois (Figura 7B). Na amostra com  $2n = 40$   
8 de Pinhão, a maioria dos cromossomos exibiu sinais de hibridação (Figura 7E). Na  
9 amostra de *E. viridans* com  $2n = 42$ , coletada na Lapa, sinais terminais foram  
10 observados em 12 cromossomos e apenas 1 exibiu sinal intersticial (Figura 6C). Na  
11 amostra com  $2n = 29$ , a maioria dos cromossomos foi detectada pela sonda, com sinais  
12 terminais, intersticiais e em cromossomos inteiros (Figura 7D).

13 Esta sonda também foi hibridada em outras amostras de *E. viridans* coletadas  
14 em Caçador/SC, Capivari do Sul/RS e Pinhão/PR, além das amostras de *E. niederleinii*  
15 coletadas em Alexandrita, Tibagi/PR, São Luis do Purunã/PR e Biturama/PR. Em  
16 nenhum destes casos foram observados sinais. Tanto para *E. viridans* quanto para *E.*  
17 *niederleinii*, a sonda hibridou somente nas amostras cujas populações estavam  
18 estruturadas, como pode ser observado no gráfico gerado pelo programa Structure  
19 (Figura 6). Aparentemente as amostras que tiveram maior coeficiente de similaridade  
20 com a amostra da sonda exibiram maior quantidade de sinais de hibridação. A única  
21 diferente foi a amostra de *E. viridans* coletada na Lapa, a qual exibiu maior quantidade  
22 de sinais do que esperado (Figura 7C), já que seu coeficiente de similaridade com o  
23 genoma que originou a sonda foi cerca de 82% (Figura 6).

24

25 **Discussão**

1 De um modo geral, as alterações cromossômicas numéricas em *Eleocharis* são  
2 provocadas por agmatoploidia, simplóidia e principalmente por poliploidia, (Da Silva *et*  
3 *al.* 2008a,b). Contudo, em subdivisões menores do gênero, como a Secção *Eleocharis*,  
4 há um conjunto maior de espécies com cariótipos derivados por agmatoploidia e  
5 simplóidia, como as séries cromossômicas encontradas em *E. uniglumis* e *E. palustris*  
6 (Bureš 1998 e Bureš *et al.* 2004). De acordo com Roalson e Friar (2000), esse grupo  
7 pode ter uma origem para e polifilética. A série *Tenuissimae* estudada aqui também  
8 pertence a esta secção e exibe uma grande variação nos números cromossômicos, como  
9  $2n = 12$  e  $2n = 20$  em *E. minima*,  $2n = 10$  em *E. retroflexa* (Da Silva *et al.* 2008a), bem como  
10 os números  $2n = 20, 29, 30, 31, 35, 40, 41$  e  $42$  encontrado aqui para *E. niederleinii* e  
11 *E. viridans*. A série *Tenuissimae* pode ter origem parafilética (Roalson e Friar 2000 e  
12 Yano *et al.* 2004), o que poderia explicar a grande diversidade morfológica das espécies  
13 desse grupo (González-Elizondo e Tena-Flores 2000).

14 As amostras mais facilmente diferenciadas, seja para *E. niederleinii* ou *E.*  
15 *viridans*, produziram inflorescências com flores férteis, aquênios viáveis, e cariótipos  
16 com menores números cromossômicos,  $2n = 20$  e  $30$  e meiose regular. Há também um  
17 caso de *E. viridans* coletada em Viamão, RS, com  $2n = 42$ , com a presença de dois  
18 cromossomos maiores em seu cariótipo, meiose regular e aquênios viáveis. Nas demais  
19 amostras prevaleceram números cromossômicos variados, a ocorrência de um a quatro  
20 cromossomos duas a três vezes maiores que os demais, derivados provavelmente de  
21 fissão/fusão nestes cariótipos. Em alguns poucos casos houve a produção de espiguetas,  
22 porém, a meiose foi irregular com formação de multivalentes e univalentes. Meiose  
23 irregular em holocêntricos, associada a rearranjos cromossômicos, foi reportada para  
24 *Carex laevigata* (Luceño e Castroviejo 1991). Além das evidências citogenéticas, a



1 sobreposição de caracteres morfológicos associada à preferência por reprodução  
2 assexuada, pode indicar uma origem híbrida para as duas espécies.

3 A FISH com a sonda de DNAr 45S revelou de 4 a 6 sítios terminais em todos  
4 os diferentes cariótipos das duas espécies. Estes sítios aparecem nos cromossomos  
5 maiores, porém não em todos eles, como observado nas amostras de *E. niederleinii* com  
6  $2n = 40$  (São Luis do Purunã e Pinhão) e de *E. viridans* com  $2n = 40$  (Lebon Regis e  
7 Pinhão). Múltiplos sítios terminais de DNAr 45S também foram encontrados em  
8 espécies de *Rhynchospora* e *Eleocharis*, sempre na posição terminal dos cromossomos  
9 (Vanzela *et al.* 1998, Da Silva *et al.* 2008a, b), mesmo quando os cariótipos sofreram  
10 rearranjos.

11 A reprodução assexuada foi observada em campo para todas as amostras  
12 estudadas aqui, inclusive para aquelas com meiose regular que floresceram em  
13 condições de casa de vegetação. Espécies prolíferas são encontradas em vários gêneros  
14 de Cyperaceae, como em *Cyperus*, *Eleocharis*, *Isolepis*, *Kyllinga* e *Schoenplectus*, e  
15 para muitos desses casos, a reprodução prolífera foi induzida por condições de  
16 ambientais estressantes. Isto foi observado em várias espécies na África do Sul  
17 (Gordon-gray *et al.* 2008). Nossos resultados não permitem concluir se a condição  
18 prolífera (reprodução assexuada) das amostras estudadas aqui aconteceu por influência  
19 do ambiente, mas é possível que este evento ocorra devido a origem híbrida, como  
20 sugerido por Chapman e Burke (2007).

21 As análises moleculares indicam uma similaridade superior a 95% entre as  
22 amostras de  $2n = 20$  e  $40$  de *E. niederleinii* coletadas em Alexandrina, isto pode  
23 explicar o padrão de marcas obtidas na GISH. Esta marcação parcial sugere a presença  
24 de mais de um genoma na composição de  $2n = 40$ , ou seja, uma origem híbrida, para  
25 esta amostra. O que poderia explicar também a ausência de inflorescências, quando

1 comparada com a amostra de  $2n = 20$ , a qual exibiu inflorescências férteis e meiose  
2 regular. Os resultados da GISH sugerem também mais de um genoma na composição  
3 dos cariótipos de *E. viridans* coletadas em Pinhão, Viamão e Irani. Alopoliplóides  
4 naturais são comuns (Soltis *et al.* 2003), e em alguns casos a GISH foi fundamental  
5 para revelar este tipo de poliploidia (Galina *et al.* 2008; Bennett *et al.* 2004), como as  
6 encontradas aqui.

7 Heilborn (citado em Davies 1956) considerou que a aparente falta de  
8 alopoliplóide em *Carex* pode ser atribuída à degeneração de três núcleos dos quatro  
9 formados ao final da meiose. Apesar desta relação não estar suficientemente esclarecida  
10 (Hoshino e Shimizu 1986), híbridos interespecíficos foram detectados em *Carex*  
11 (Luceño 1994).

12 Apesar da taxonomia identificar duas espécies (*E. niederleinii* e *E. viridans*), as  
13 análises moleculares indicaram sete genomas estruturados com algumas sobreposições,  
14 e as análise de hibridação genômica, mais de dois genomas. Essas informações nos  
15 permitem sugerir a existência de isolamento, mesmo em situação simpátrica, sobretudo  
16 para as amostras de Pinhão. Nossos resultados abrem uma excelente perspectiva para  
17 estudos futuros envolvendo análises populacionais, de marcadores de herança  
18 extragenômica, hibridação e poliploidia, e assim contribuir para o esclarecimento das  
19 relações de parentesco dentro da série *Tenuissimae*.

20

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1 **Legendas**

2 **Figura 1.** Mitose em *Eleocharis niederleinii*, com diferentes números cromossômicos.

3 A) Amostra com  $2n = 20$ , com os cromossomos tamanhos próximos. B)  
4 Amostra com  $2n = 29$ . Note o núcleo arreticulado à esquerda. C) Amostra com  
5  $2n = 30$ , com os cromossomos tamanhos próximos. D) Amostra com  $2n = 30$ .  
6 E) Amostra com  $2n = 31$ . F) Amostra com  $2n = 35$ , com os cromossomos  
7 tamanhos próximos. G - I) Amostras com  $2n = 40$ . Todas as setas apontam os  
8 cromossomos maiores. Barra =  $10\mu\text{m}$

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10 **Figura 2.** Mitose em *Eleocharis viridans*, com diferentes números cromossômicos. A)

11 Amostra com  $2n = 20$ , com os cromossomos tamanhos próximos. B) Amostra  
12 com  $2n = 29$ . C) Amostra com  $2n = 31$ . D e E) Amostras com  $2n = 40$ . F e G)  
13 Amostras com  $2n = 41$ . G) Amostra com  $2n = 42$ . Todas as setas apontam os  
14 cromossomos maiores. Barra =  $10\mu\text{m}$ .

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16 **Figura 3.** Diferentes fases da meiose para *E. niederleinii* e *E. viridans*. A) Metáfase I

17 em *E. niederleinii* com  $2n = 20$  e 10 bivalentes. B) Metáfase I em *E.*  
18 *niederleinii* com  $2n = 30$  e 15 bivalentes. C) Metáfase I em *E. viridans* com  $2n$   
19  $= 42$  e 21 bivalentes. Note os dois bivalentes maiores. D) Metáfase I em *E.*  
20 *niederleinii* com  $2n = 30$  e com  $1^{\text{IV}} + 2^{\text{III}} + 9^{\text{II}} + 2^{\text{I}}$ . E) Metáfase I em *E.*  
21 *viridans* com  $2n = 31$  e  $7^{\text{III}} + 3^{\text{II}} + 4^{\text{I}}$ . F) Metáfase I em *E. viridans* com  $2n =$   
22  $31$  e  $1^{\text{VI}} + 1^{\text{IV}} + 5^{\text{III}} + 2^{\text{II}} + 2^{\text{I}}$ . G) Metáfase I em *E. viridans* com  $2n = 41$  e  $1^{\text{III}} +$   
23  $19^{\text{II}}$ . E m todas estas fotos: o “I” indica os univalentes, as setas apontam as  
24 associações multivalentes e as cabeças de seta apontam as associações  
25 trivalentes. H) Metáfase polínica em *E. niederleinii* com  $n = 15$ , note os dois



1 cromossomos maiores. I) Metáfase polínica em *E. niederleinii* com  $n = 17$ , e  
2 todos os cromossomos com tamanhos próximos. J e K) Metáfase polínica em  
3 *E. viridans* com  $n = 22$  e  $n = 21$ , respectivamente, as setas apontam os  
4 cromossomos maiores. Barra =  $10\mu\text{m}$ .

5

6 **Figura 4.** Hibridação *in situ* com a sonda de DNAr 45S em amostras de *E. niederleinii*.

7 A) Amostra com  $2n = 20$  e quatro sinais de hibridação. B) Amostra com  $2n =$   
8  $29$  e quatro sinais de hibridação. C) Amostra com  $2n = 30$  e seis sinais de  
9 hibridação. D) Amostra com  $2n = 30$  e seis sinais de hibridação. E) Amostra  
10 com  $2n = 31$  e seis sinais de hibridação. F) Amostra com  $2n = 40$  e quatro  
11 sinais de hibridação. Note que nas figuras D, E e F, os dois cromossomos  
12 maiores portam os sítios de DNAr 45S. G) Amostra com  $2n = 40$  e quatro  
13 sinais de hibridação. H) Amostra com  $2n = 35$  e seis sinais de hibridação. I)  
14 Amostra com  $2n = 40$  e seis sinais de hibridação. A seta aponta o único  
15 cromossomo maior sem o sítio de DNAr 45S. Barra =  $10\mu\text{m}$ .

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17 **Figura 5.** Hibridação *in situ* com a sonda de DNAr 45S em amostras de *E. viridans*. A)

18 Amostra com  $2n = 20$  e quatro sinais de hibridação. B) Amostra com  $2n = 29$  e  
19 seis sinais de hibridação. C) Amostra com  $2n = 31$  e seis sinais de hibridação.  
20 D) Amostra com  $2n = 40$  e quatro sinais de hibridação. E) Amostra com  $2n =$   
21  $40$  e seis sinais de hibridação. F) Amostra com  $2n = 41$  e seis sinais de  
22 hibridação. Nas figuras D, E e F, as setas apontam os cromossomos maiores  
23 sem o sítio de DNAr 45S. G) Amostra com  $2n = 42$  e seis sinais de hibridação.  
24 H) Amostra com  $2n = 41$  e quatro sinais de hibridação. Barra =  $10\mu\text{m}$ .

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1 **Figura 6.** A) Mapa com os pontos de coleta. Os números representam as exsiccatas de  
2 cada amostra. Os números vermelhos são de *E. niederleinii* os pretos são de *E.*  
3 *viridans*. Note que apenas em um ponto as duas espécies foram coletadas  
4 juntas (Pinhão). B) Dendrograma de similaridade genética obtido pelo  
5 coeficiente de Jaccard e pelo método UPGMA para as duas espécies.

6  
7 **Figura 7.** Hibridação genômica *in situ*. A) Amostra de *E. niederleinii* com  $2n = 40$   
8 coletada em Alexandrita/MG, hibridada com a sonda da amostra de *E.*  
9 *niederleinii* com  $2n = 20$  coletada no mesmo local. As fotos de B – E, são de  
10 diferentes amostras hibridadas com a sonda de *E. viridans* com  $2n = 20$   
11 coletada em Viamão/RS. B) Amostra de *E. viridans* com  $2n = 42$  coletada em  
12 Pinhão/PR. C) Amostra de *E. viridans* com  $2n = 42$  coletada na Lapa. D)  
13 Amostra de *E. viridans* com  $2n = 29$  coletada em Irani/SC. Amostra de *E.*  
14 *viridans* com  $2n = 40$  coletada em Pinhão/PR. Barra = 10 $\mu$ m.

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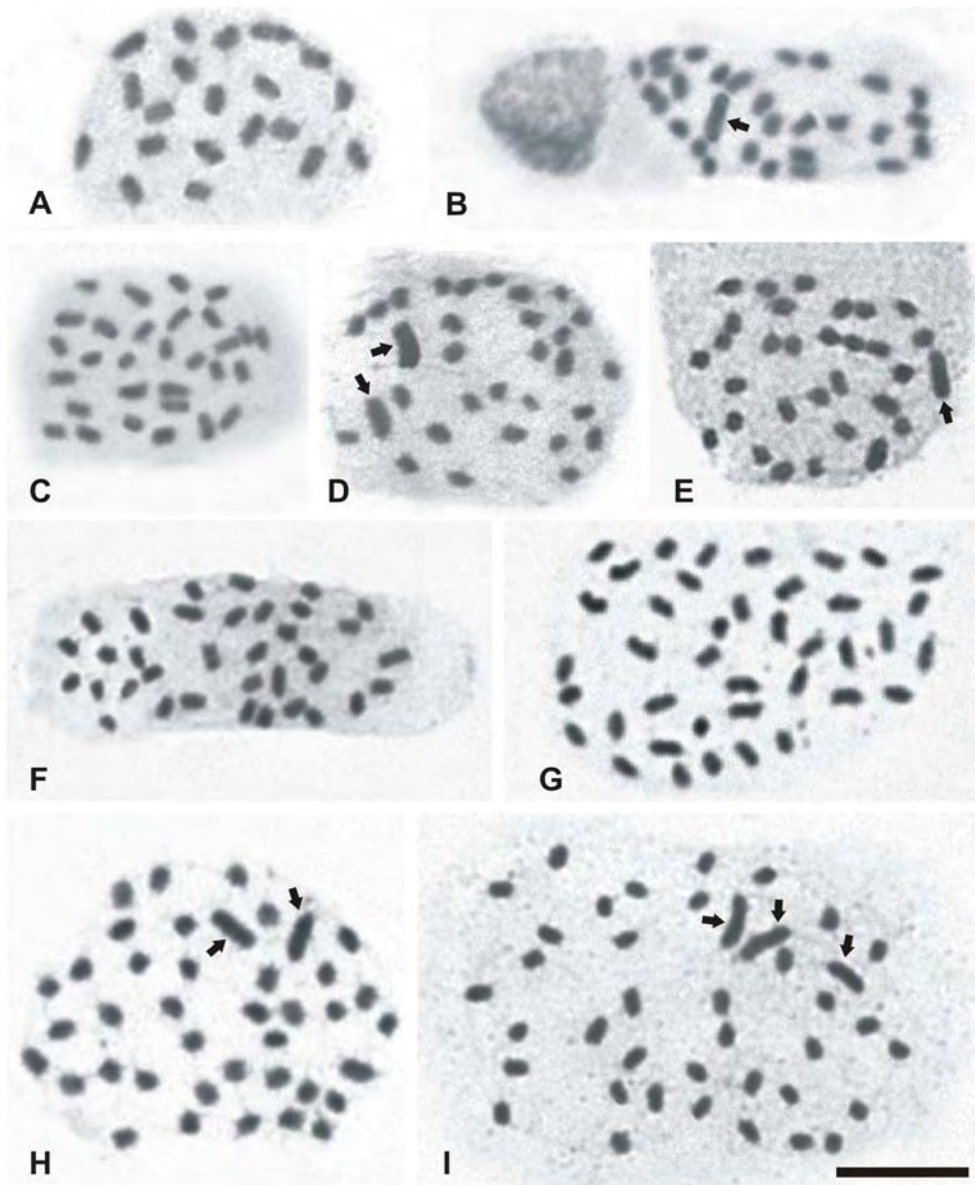
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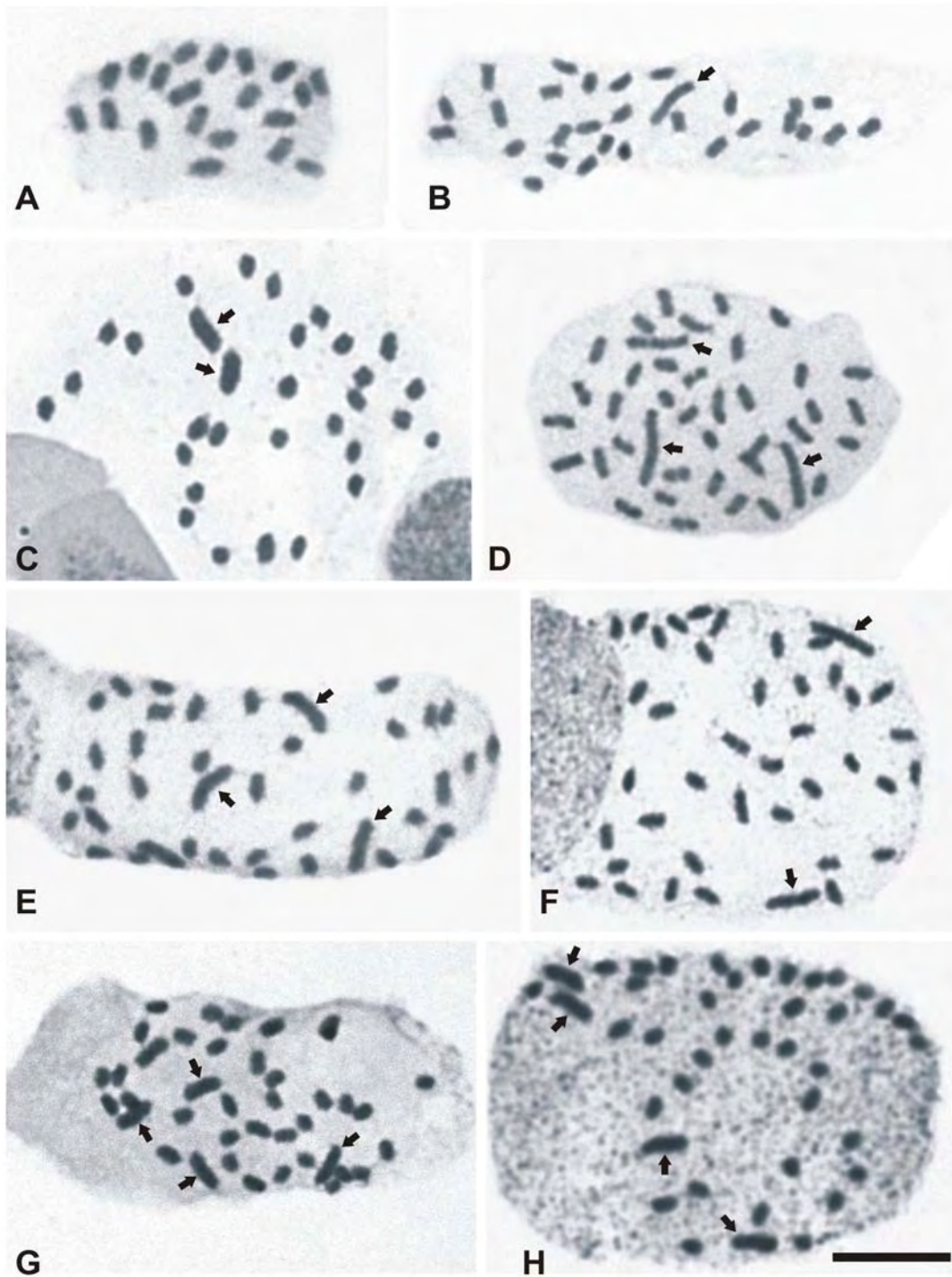
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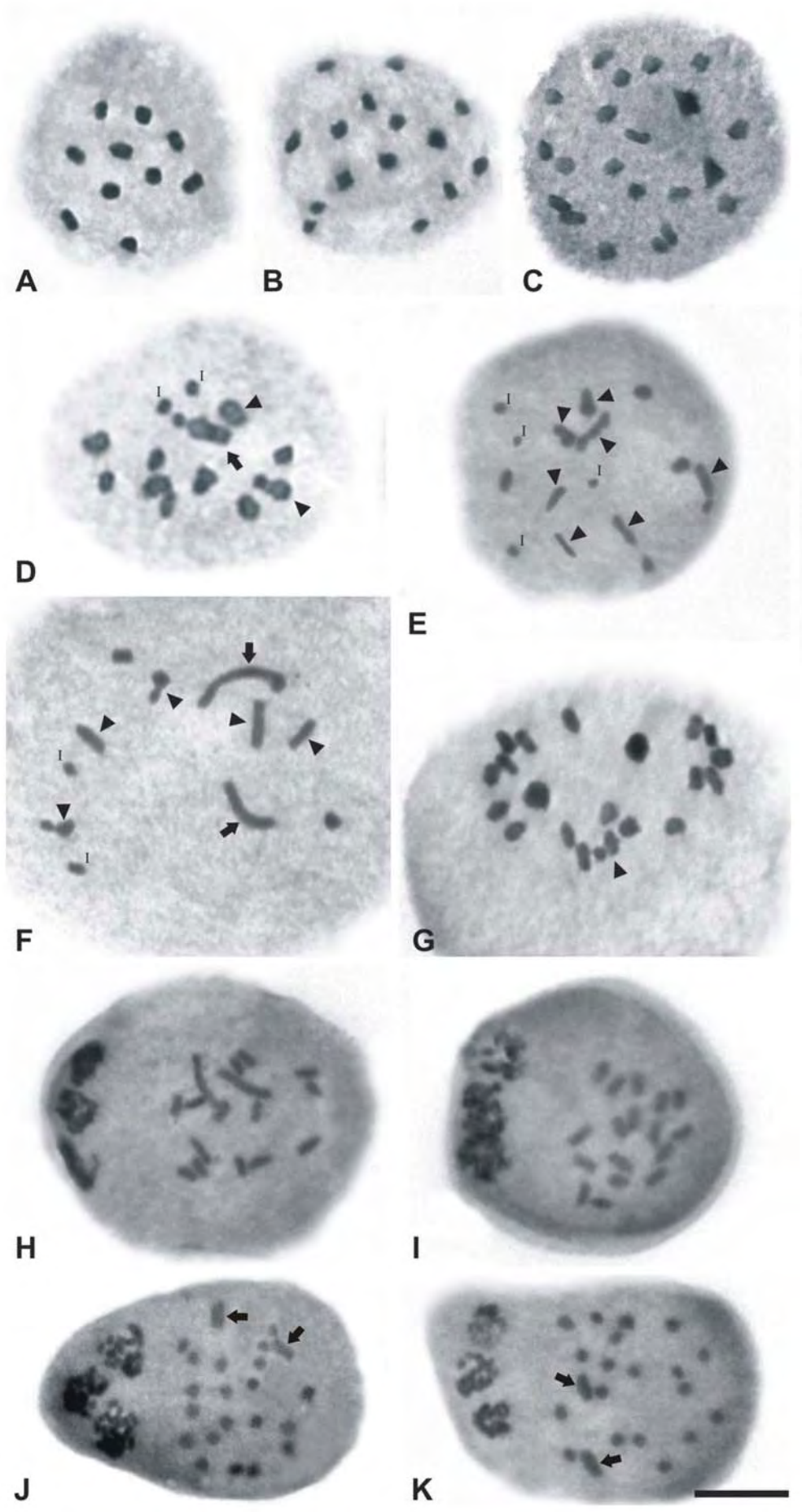
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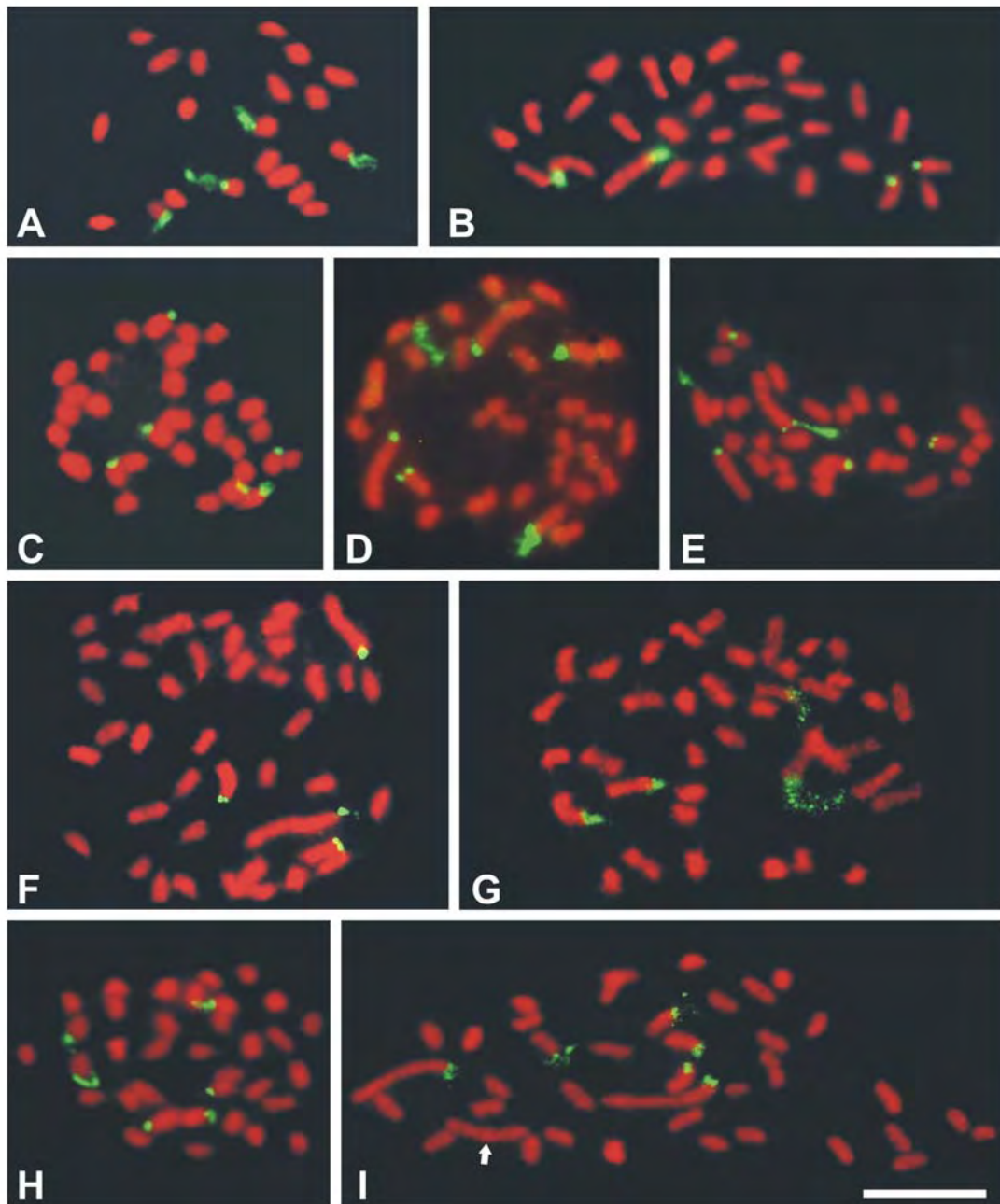


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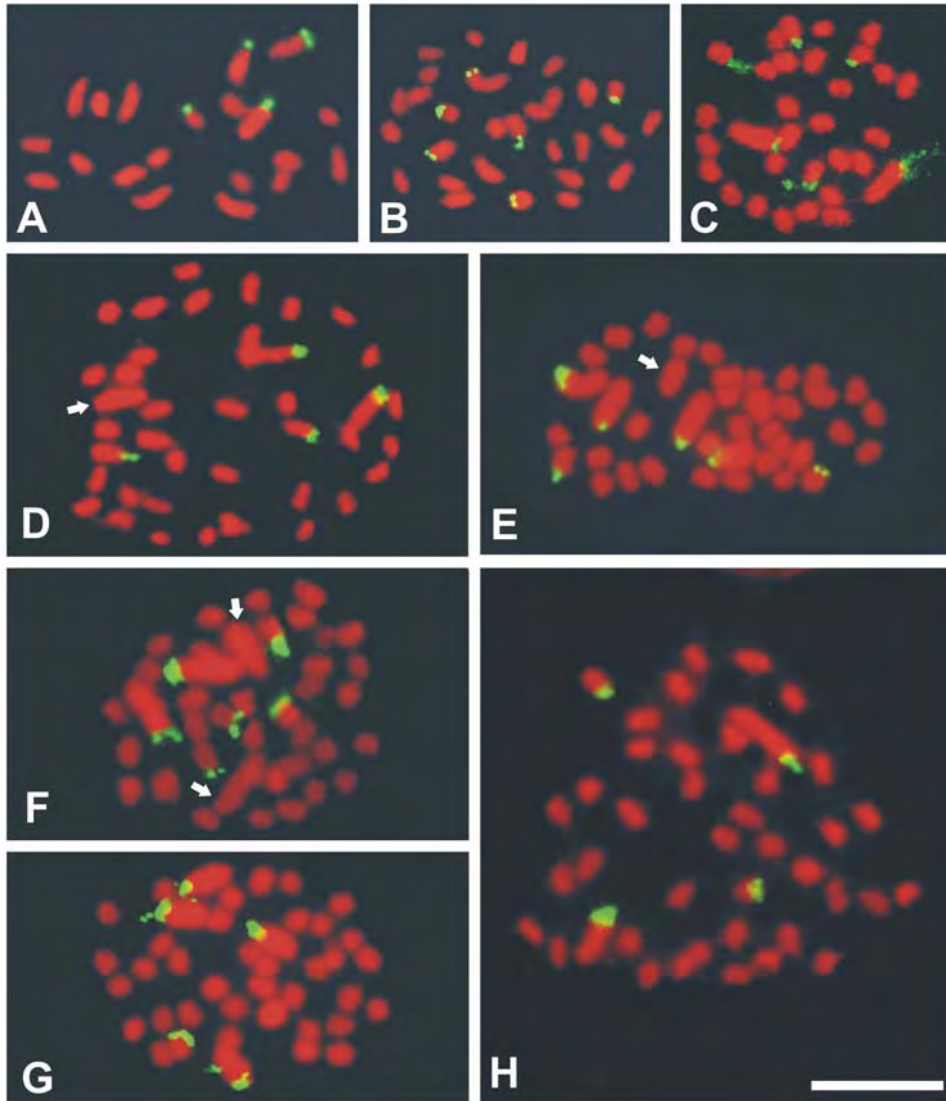


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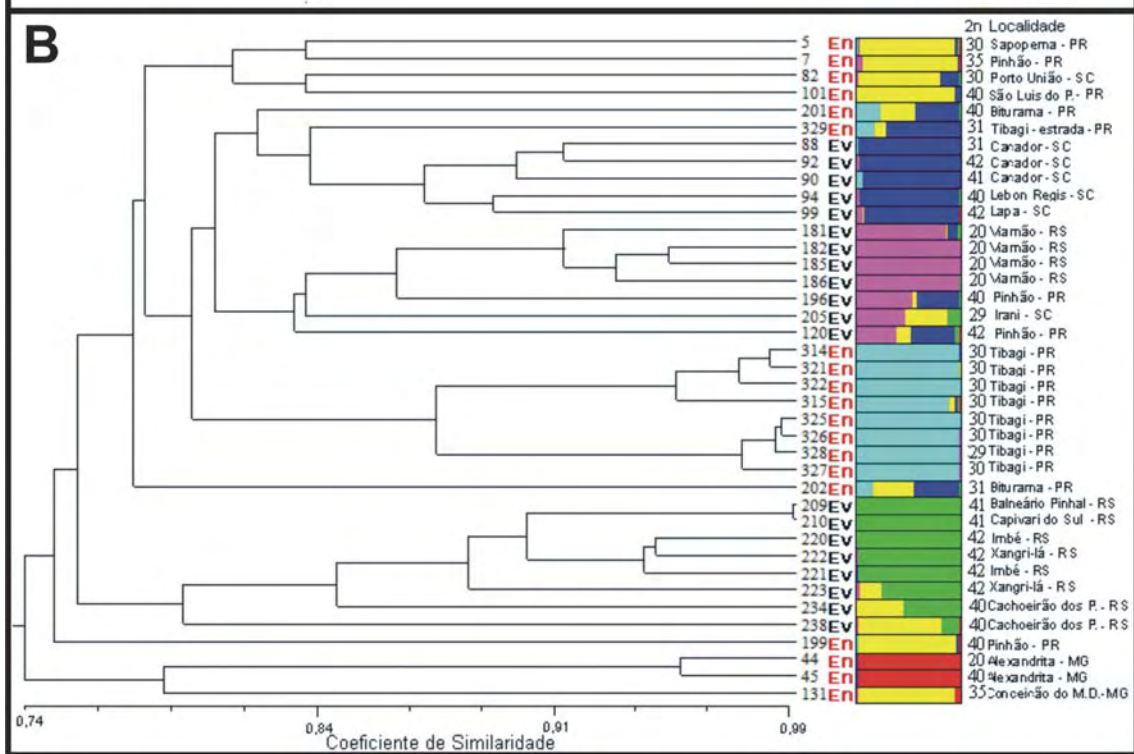
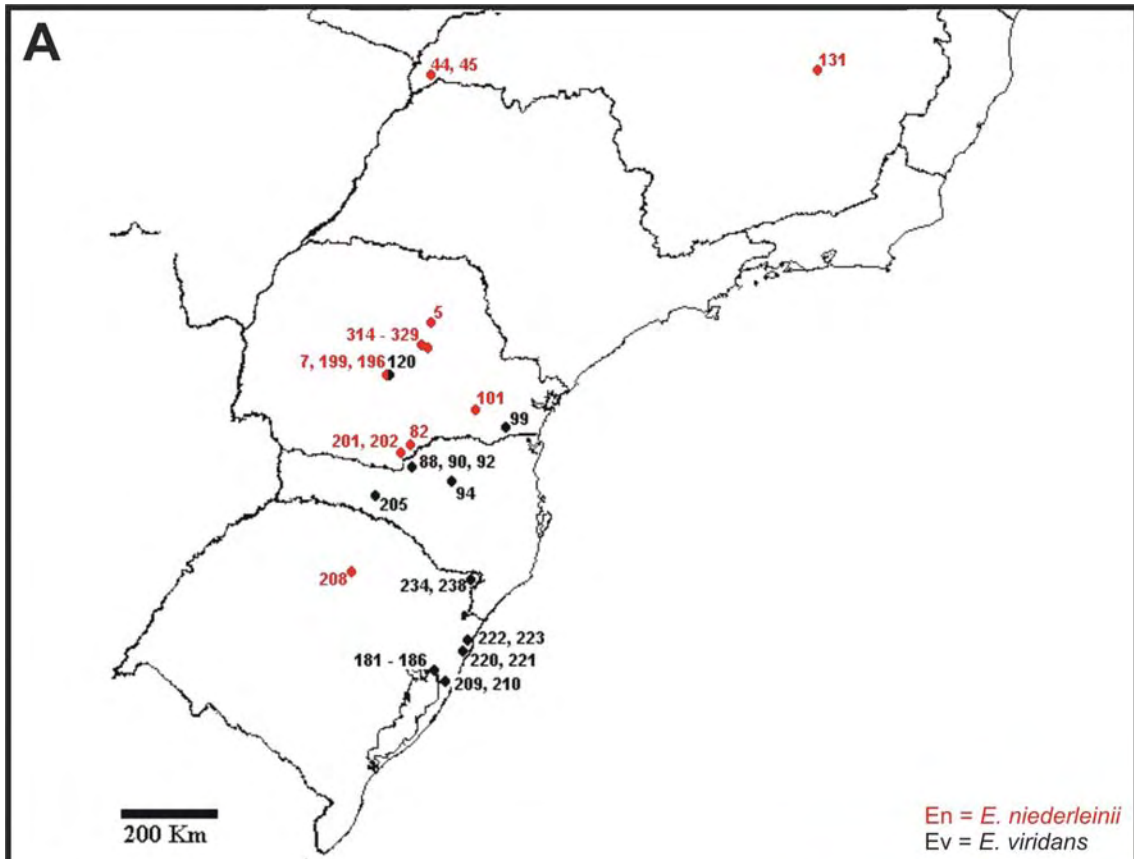




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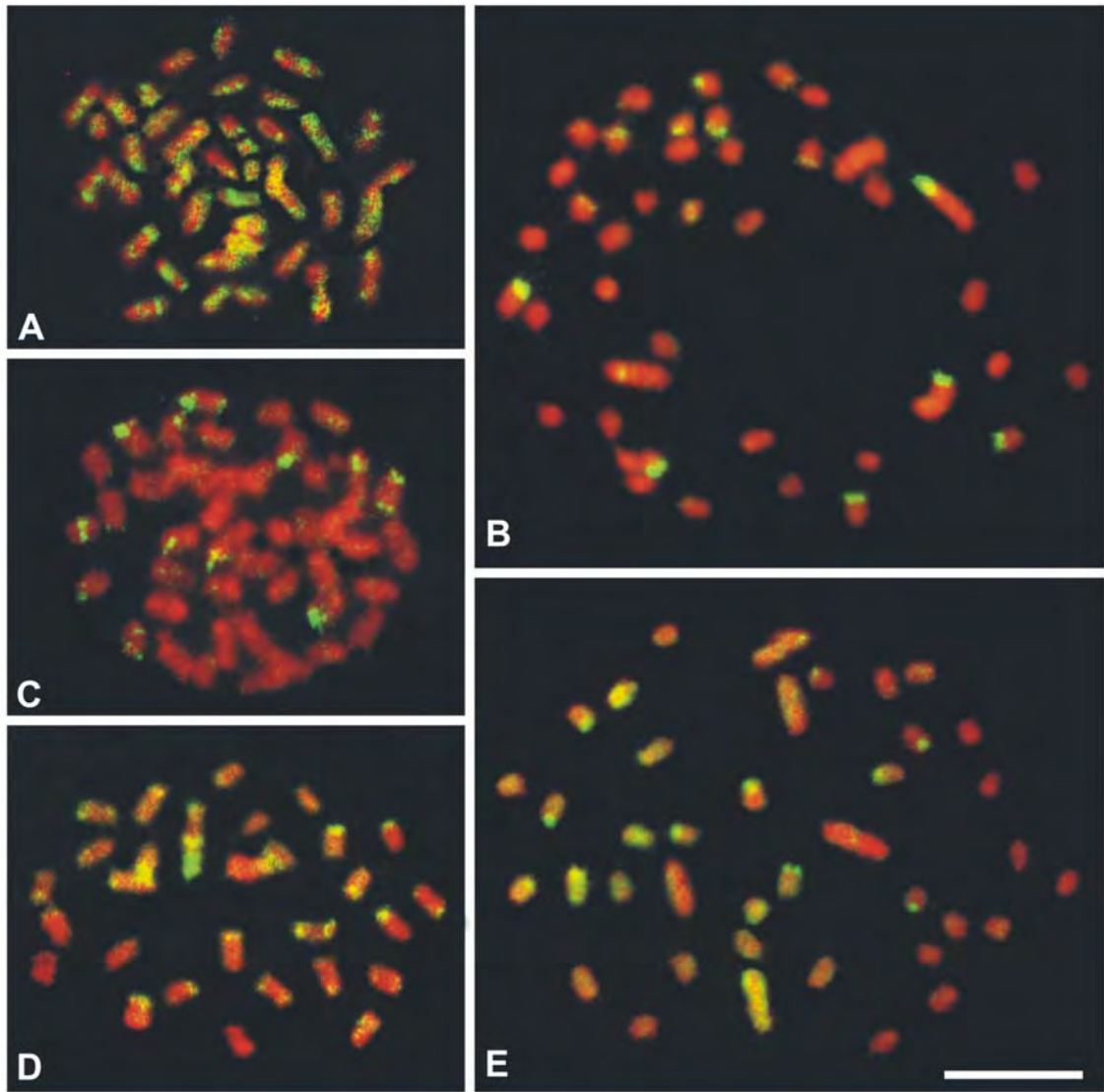


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40(3)*	76.12	4.63 3.96 3.93 4.88 4.67 4.34 4.28	26°49'67"S 50°21'54"W, Lebon Regis, SC (94)
40(4)*	80.96	4.67 4.34 4.28	25°39'59"S 51°40'05"W, Pinhão, PR (196) Caminho do Cachoeirão dos Padrinhos – RS (234) Caminho do Cachoeirão dos Padrinhos – RS (238)
41(2)*	79.57	4.69 4.23	26°51'31"S 50°49'54"W, Caçador, SC (90)
41(4)*	81.22	4.65 4.23 4.22 4.19	30°12'08"S 50°12'52"W, Balneário Pinhal, RS (209)** 30°09'15"S 50°25'53"W, Capivari do Sul, RS (210)**
42(4)*	81.72	4.74 4.73 4.37 4.36	25°36'47"S 49°49'55"W, Lapa, PR (99)** 26°51'31"S 50°49'54"W, Caçador, SC (92)** 25°39'59"S 51°40'05"W, Pinhão, PR (120)** 29°54'18"S 50°06'15"W, Imbé, RS (220)** 29°54'18"S 50°06'15"W, Imbé, RS (221)** 29°48'18"S 50°03'45"W, Xangri-lá, RS (222)** 29°48'18"S 50°03'45"W, Xangri-lá, RS (223)**

<sup>A</sup> Comprimento total do lote diplóide em  $\mu\text{m}$ . <sup>B</sup>: Tamanho dos cromossomos maiores em  $\mu\text{m}$ . \*Entre parênteses a quantidade de cromossomos muito maiores que os demais. \*\* plantas que produziram inflorescência.

## **Conclusões**

## Conclusões

1) Nenhuma das 27 amostras estudadas exibiu constrição primária. Somente constrições nucleolares foram observadas em algumas metáfases para poucas espécies. Em *E. sellowiana* e *E. maculosa* a migração paralela dos cromossomos foi registrada. Para várias espécies, bivalentes formando a “estrutura em caixa” puderam ser observados. Assim, os dados resultantes deste trabalho confirmam a condição holocêntrica dos cromossomos neste gênero.

2) As medições cromossômicas feitas em 25 espécies mostram diminuição gradual no tamanho para a maioria delas. Com exceção, de *E. maculosa*, *E. niederleinii*, *E. viridans* e *E. subarticulata*, as quais exibiram cromossomos muito maiores em relação aos demais do seu cariótipo. Nas três primeiras espécies estes cromossomos grandes, surgiram por simploidia, e na última espécie, translocações múltiplas pode ser a causa da formação destes cromossomos.

3) Todas as espécies do subgênero *Limnochloa* estudadas exibiram  $2n = 40$  ou mais cromossomos e estes foram menores que  $1,4 \mu\text{m}$ .

4) Os números cromossômicos variaram de  $2n = 6$  (*E. subarticulata* e *E. maculosa*) até  $2n = 60$  (*E. laeviglumis*), com vários números intermediários, como 10, 20, 30 e 40, para a maioria das espécies. A alta frequência de espécies com números cromossômicos múltiplos de 5, concordam com trabalhos anteriores (Löve e Love 1957; Da Silva *et al.* 2008) que indica  $x = 5$  como o número básico do gênero.

5) Apesar de *E. maculosa*, apresentar mais de um número cromossômico, decorrente de simploidia (fusão), e *E. niederleinii* e *E. viridans*, exibirem uma série cromossômicas causada provavelmente por poliploidia e rearranjos cromossômicos (fusão/fissão) associada a hibridação. A maioria da espécie (cerca de 80%) foi poliplóide. Porém não foi possível concluir se ocorre há predominância de auto ou alopoliplóides.

6) Regiões heterocromáticas ricas em CG (DAPI<sup>-</sup>/CMA<sup>+</sup>) foram localizadas apenas nas pontas dos cromossomos. Em várias espécies estas regiões estão associadas aos sítios de DNAr 45S.

7) Os sítios de DNAr 45S variaram em número entre as espécies, muitas vezes não acompanhando o nível de ploidia, como visto em *E. flavescens* ( $2n = 10$ ) com 10 sítios e em *E. bonariensis* ( $2n = 20$ ) com apenas dois. Mesmo quando os cariótipos sofreram rearranjos, como em *E. maculosa* com  $2n = 10, 8, 7$  e  $6$ , o número de sítios foram sempre quatro. Em todas as espécies analisadas os sítios foram terminais. Nas espécies que tiverem cariótipos mais simétricos, mais sítios de DNAr 45S foram encontrados. Estes dados nos permitem concluir que rearranjos cromossômicos (fusão, fissão e poliploidia) têm pouca influência na dispersão destes sítios, e que esta multiplicação de sítios pode ser resultado de amplificação seguido de dispersão pelas pontas dos cromossomos com tamanho próximos.

8) Os dados citológicos mostrados neste trabalho corroboram os de Yano *et al.* (2004) e Roalson e Friar (2000), os quais separam as espécies do subgênero *Limnochloa* dos demais subgêneros. Nossos resultados mostram que as espécies de *Limnochloa*, possuem

cariótipos com cromossomos numerosos e pequenos ( $< 1,4 \mu\text{m}$ ). Os demais subgêneros possuem cromossomos maiores ( $> 1,5 \mu\text{m}$ ). Assim sendo, a análise citogenética pode ser utilizada neste caso como um caráter micromorfológico muito útil na taxonomia.

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