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Evidence of genetic differentiation and karyotype evolution of the sedges *Cyperus ligularis* L. and *C. odoratus* L. (Cyperaceae)

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ABSTRACT

The taxonomy of Cyperaceae is complex, with genera like *Cyperus* harboring species complexes. We analyzed the genetic similarity between *Cyperus ligularis* L. and *C. odoratus* L. based on DNA fingerprinting and cytogenetics. Significative genetic differentiation (G_{ST} = 0.363) and low gene flow (N_m = 0.877) indicated a clear genetic distinction between the two species. Moreover, the clustering analysis showed two distinct genetic groups, suggesting a lack of evidence for hybridization. The phenogram revealed two different lineages, and although all individuals of *C. odoratus* were collected from plots close to each other, they possessed greater genetic diversity than that observed among individuals of *C. ligularis*, which were sampled over a wider geographic range. Variation in chromosome number within the two species exhibited the opposite pattern, indicating greater karyotype stability in *C. odoratus* with 2*n* = 72 and 2*n* = 76, while the diploid number for *C. ligularis* varied from 2*n* = 66 to 88. The lower genetic variation in *C. ligularis* may be a result of the founder effect associated with seed dispersion and clonal reproduction. Field observations and analysis of reproductive biology should enrich the understanding of the genetic structure of the investigated populations and their role in successional processes.

Keywords: agmatoploidy, DNA amplification fingerprinting, gene flow, genetic differentiation, hybridization

Introduction

The family Cyperaceae (sedges) presents several taxonomic uncertainties with the presence of some species complexes, most of them described for *Carex* spp., such as *Carex flava* and *Carex macrocephala* species complexes (Hedrén 2004; King & Roalson 2009; Jiménez-Mejías *et al*. 2014), among others. For other Cyperaceae genera, there are few records of species complexes as seen in *Rhynchospora* (e.g., *Rhynchospora glomerata*) (Naczi & Moyer 2017). *Cyperus*, which is among the largest genera of the family, is considered complex, with most of the controversial groups inhabiting tropical regions (Simpson *et al*. 2003), mainly in Brazil, with about 100 species registered in varied vegetation types (Alves *et al*. 2015). In the most recent phylogenetic approaches, based on morphology and molecular data (Larridon *et al*. 2013; Reid *et al*. 2014), *Cyperus* was not recovered as a monophyletic group, being divided in the monophyletic clade "*Cyperus* C4" and the paraphyletic clade "*Cyperus* C3". The complexity of the genus *Cyperus* is also perceived at the intraspecific relationships level, as seen, for instance, in the *C. rigens* group (Reid *et al*. 2014).

It has been suggested that some degree of cross-species hybridization might occur between sympatric populations of the widely distributed group of articulated spikes, which

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comprises highly variable species as *C. ligularis* and *C. odoratus* (Luceño & Alves 1997). However, analyzing the internal anatomy of leaves of these two species, Martins & Alves (2009) did not find any evidence of hybridization. Species delimitation analyses in Cyperaceae have elucidated intraspecific relationships within difficult genera by accessing DNA fingerprinting profiles and karyotype features, as in the case of species of *Carex* and *Schoenoplectus* (Hipp *et al*. 2007; Esselman *et al*. 2012; Gizaw *et al*. 2016). Therefore, we relied on a genetic characterization of sympatric individuals of both *C. ligularis* and *C. odoratus*, by means of DNA Amplification Fingerprinting (DAF) and cytogenetic analyses, to access their diversity and the relationship between both species.

Materials and methods

Plant material

For molecular analysis, young leaves were collected from 40 individuals of *Cyperus odoratus* L. and *C. ligularis* L. populations, 20 from each, in five collection plots in open spaces and on the border of an urban fragment of the Atlantic Forest fragment (Tab. 1), with a minimum distance of three meters among individuals (Fig. 1). For cytogenetic analyses, young root tips of both species were collected, pre-treated with 2 mM 8-hidroxiquinolein for 4 h at 18 °C, and then fixed in ethanol: acetic acid (3:1) for 4-6 h at room temperature (ca. 25 °C).

Molecular data analyses

DNA extraction procedures followed the CTAB protocol as described by Weising *et al*. (2005), with minor modifications on the tissue amount (2 g to 1 g of fresh leaves) and the volume of the extraction buffer (10 ml to 12 ml). The DAF analysis was carried out following Caetano-Anollés *et al*. (1991) with modifications introduced by Winter *et al*. (2000) and Benko-Iseppon *et al*. (2003). The PCR reactions, with a final volume of 15 μL, consisted of 20 ng of DNA template, 10× PCR buffer, 2.5 mM $MgCl_2$, 10 mM dNTP-mix (Fermentas), 20 μM primer (Tab. S1 in supplementary material) and 1.0 U *Taq* polymerase (Fermentas). The DNA amplifications were performed in an Eppendorf Mastercycler Gradient thermocycler, using a program consisting of 2 min initial denaturation step (95 °C), followed by 40 cycles of 15 s at 95 °C, 1 min at 35 °C and 2 min at 72 °C, and the reactions were completed by a final extension step of 7 min at 72 °C. PCR products were separated by electrophoresis on 1.8 % agarose gel and stained with 0.5 μg/mL ethidium bromide.

The selection of the most informative primers was performed from an initial set of 50 DAF primers (Tab. S1 in supplementary material) tested on four random individuals (two of each species), using as parameters the number of amplified loci and polymorphic bands. The 10 most informative primers were used to amplify the DNA of the 40 individuals of both species. The genotyping process resulted in a binary matrix of presence (1) and absence (0) of amplified DNA fragments above 100 pb.

In order to check the relationships between the two species, the genetic dissimilarity analysis was performed according to the Jaccard's coefficient (Perrier *et al*. 2003) using the program DARwin 6.0 (Perrier & Jacquemoud-Collet 2006), to generate a phenogram based on the weighted neighbor-joining algorithm (2000 replications of bootstrap). The genetic differentiation between the two species (here represented by two populations) was accessed by G_{ST}, a statistical index which is appropriate to analysis where the contribution of genetic drift among populations differences is not of interest (Holsinger & Weir 2009). Moreover, according to Wright (1931), the genetic differentiation among populations is directly related to

Table 1. Sampled of individuals of *Cyperus ligularis* and *C. odoratus* and their georeferenced locations in Recife, Pernambuco, Brazil. Abbreviation of the plot collections: Açude da Prata – AP, Estrada dos Passarinhos – EP, Ilha do Bananal – IB, Distrito de Apipucos – API and Fábrica da Macaxeira – FM.

Species	Number of individuals	Collection site	Environmental features	Geographic coordinates
Cyperus odoratus (Cyp 001)	20	Border of urban forest remaining (Açude da Prata – AP)	Region exposed to very humid flooding during rainy season	8°00'24.15"S 34°56'57.23"W
Cyperus ligularis (Cyp 002)	6	Açude da Prata – AP	Region exposed to very humid flooding during rainy season	8°00'24.15"S 34°56'57.23"W
	3	Border of urban forest remaining (Estrada dos Passarinhos – EP)	Region perturbed by urban occupation and burning activities	8°00'50.18"S 34°56'29.07"W
	2	Open space (Ilha do Bananal – IB)	Island surrounded by the river Capibaribe characterized by grazing activities	8°01'30.76"S 34°56'08.61"W
	7	Open space (Distrito de Apipucos – API)	Locality perturbed by urbanization	8°01'05.84"S 34°55'50.88"W
	2	Open space (Fábrica da Macaxeira – FM)	Locality perturbed by grazing activities	8°00'51.12"S 34°55'53.74"W

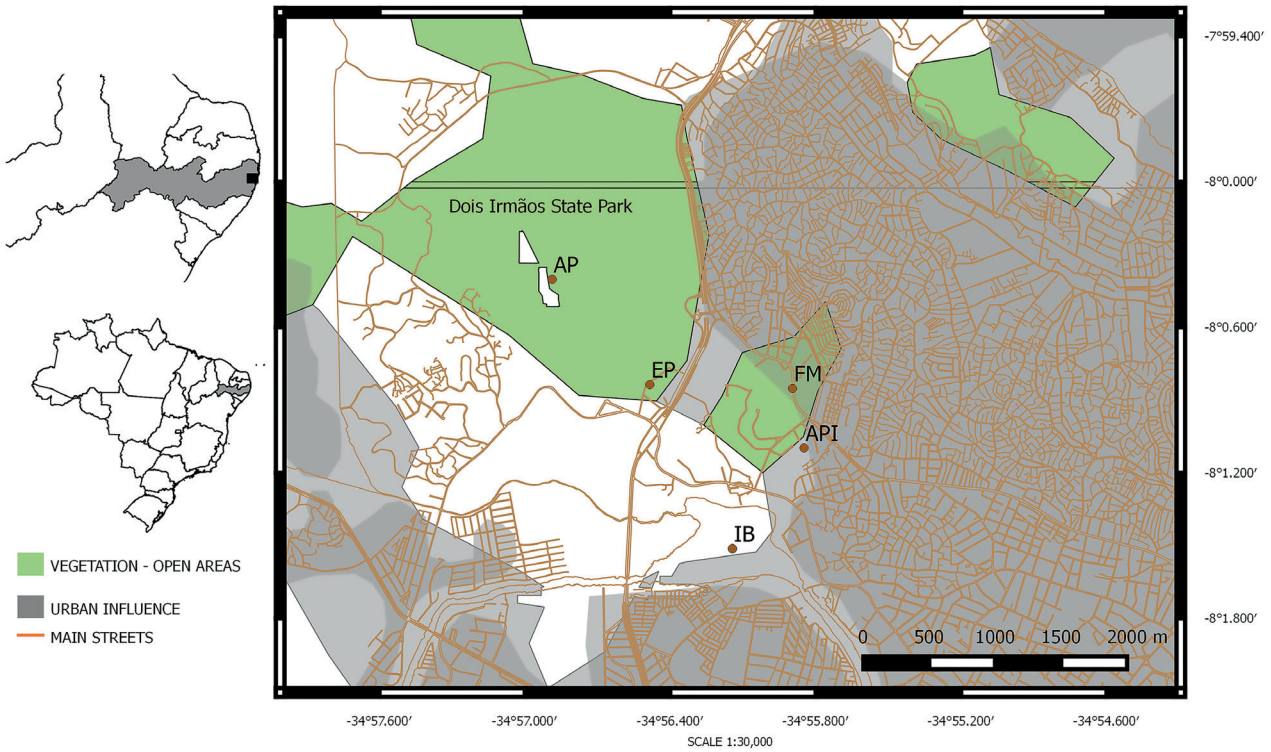

Figure 1. Plots collection of *C. odoratus* and *C. ligularis* located within and around the Dois Irmãos State park. Occurrence of both species – Açude da Prata – AP; Occurrence only of *C. ligularis* – Estrada dos Passarinhos – EP; Fábrica da Macaxeira – FM; Distrito de Apipucos – API; Ilha do Bananal – IB.

the migration, which was accessed through the number of migrants (N_m). The G_{ST} and N_m were calculated using the Popgene 3.2 program (Yeh & Boyle 1997) based on 1000 simulations. The genetic clusters were verified to assure whether there is evidence of interspecific hybridization between the two species. For this purpose, the software Structure 2.3.3 (Pritchard *et al*. 2000) was used through the Bayesian method where the K value was identified and analyzed to obtain the level of genetic admixture between the two species. The interaction between individuals was measured based on 600.000 replicates (60.000 in the burn-in stage), using the admixture model. The number of genetic clusters was inferred through the index described by Evanno *et al*. (2005).

Cytogenetic analyses

A total of 15 individuals of the two populations were analyzed, nine of *C. ligularis* and six of *C. odoratus*. Slides were prepared as described by Benko-Iseppon & Morawetz (2000), with modifications. Root tips were digested in an aqueous solution of 2 % cellulose (Calbiochem 21947) and 20 % pectinase (Sigma) for 90 min at 37 °C. Meristems were squashed in 45% acetic acid and frozen in liquid nitrogen to remove the coverslip. Then, slides were aged for three days at room temperature, stained with CMA (0.5 mg/mL, 1 h) and DAPI (2 µg/mL, 30 min), mounted in McIlvaine's buffer (pH 7.0): glycerol (1:1, v/v) and stored for another three days, according to Schweizer & Ambros (1994). After image capture of ca. 100 cells of each species (with a Leica DMLB epifluorescence microscope and an Olympus SP350 camera), slides were destained in ethanol:acetic acid (3:1, v/v) for 30 min, followed by immersion in ethanol for 1 h. Then, chromosome preparations were hydrolyzed in 5 N HCl at room temperature for 20 min and stained with 2 % Giemsa for 15 min and mounted with Entellan (Merck), for further image acquisition. Silver nitrate ($AgNO_3$) impregnation of interphase nuclei followed the protocol described by Hizume et al. (1980), with the modifications described in Vasconcelos *et al*. (2010). Thirty microliters of 50% silver nitrate were added to the preparations, which were covered with a piece of nylon mesh (24×32 cm), incubated at 37 °C for 40 min in a moisture chamber, being subsequently washed in distilled water and mounted with Entellan. For the chromosome counting and the silver nitrate impregnation approaches, at least 100 and 1000 cells were analyzed per species, respectively.

Results and discussion

A total of 172 polymorphic bands were observed at intra- and interspecific levels (Tab. 2). The *C. odoratus* population presented the highest polymorphism level, represented by 124 polymorphic loci (Tab. 2). According

Table 2. Number of polymorphic loci at the intra- and interspecific levels, considering the ten selected DAF primers applied in *Cyperus ligularis* and *C. odoratus*.

Primer	Sequence (5'-3')	Intraspecific polymorphism		Interspecific polymorphism	Total polymorphism
		Cyperus ligularis	*Cyperus odoratus*		
15_1	TGCGTGCTTGTATAA	7	17	11	18
15_4	TGCGTGCTTGATATA	8	13	16	16
15_5	TGCGTGCTTGAGAGA	3	10	17	17
15_6	TGCGTGCTTGAAGAA	7	12	14	14
15_8	TGCGTGCTTGTTAAT	1	15	16	16
15_9	TGCGTGCTTGTTCAT	3	10	14	14
15_10	AGGTCTTGGGTATAA	6	12	14	14
15_12	AGGTCTTGGGTAGGC	21	14	28	28
15_14	GTGGGCTGACGTGGG	6	16	18	18
OPJ-16	TCTCCGCCCT	12	5	17	17
–	Total	74	124	165	172

to Hollingsworth & Ennos (2004), for a reliable estimation of genetic diversity with dominant markers, at least 50 polymorphic loci are necessary for both resolving tree topologies and differentiating populations. The authors conclude that there is a direct relationship between the consistency of the genetic diversity analysis and the number of obtained polymorphic loci. Therefore, our results for the two analyzed *Cyperus* species are well supported, considering the relatively high number of sampled loci, despite the low number of sampled individuals.

Genetic dissimilarity and clustering analyzes

As previously mentioned, there are evidences of natural interspecific hybridization among sympatric populations of related *Cyperus* species (e.g., *C. iria* and *C. microiria*; Chozin & Yasuda 1991), as suggested for *C. odoratus* and *C. ligularis* by Luceño & Alves (1997). Therefore, considering this hypothesis, we analyzed the genetic dissimilarity between these two species.

The analysis of population genetic differentiation (G_{ST}) and gene flow (N_m) between the sampled species revealed a clear differentiation between the two species, with G_{ST} = 0.363 and N_m = 0.877, considering that values of $G_{ST} > 0.25$ (Wright 1978) and $N_m < 1$ indicate a favorable scenario for genetic differentiation (Almeida *et al*. 2003). Moreover, the Bayesian clustering analysis also indicated no evidence of hybridization between the species, although some individuals of *C. ligularis* and *C. odoratus* presented subtle evidences of ancestral mixing, which also may be associated with the highly polymorphic nature of the DAF markers. Interestingly, three genetic clusters were observed, being two distinct groups between *C. odoratus* and *C. ligularis* (Fig. 2). In addition, the neighbor-joining phenogram (Fig. 3) showed two main clades clearly separating *C. odoratus* and *C. ligularis* in different clusters, confirming the ITS-based molecular phylogeny by Reid *et al*. (2014) and the internal anatomy analysis by Martins & Alves (2009), respectively.

Regarding the intraspecific relationships, the individuals of *C. odoratus* presented a higher level of differentiation when compared with individuals of *C. ligularis* (Tab. 2). The clustering data also makes this clear, where *C. odoratus* presents two predominant genetic clusters (Fig. 2 – red and blue clusters) showing a higher level of genetic differentiation. Moreover, a higher level of genetic differentiation was also observed in the neighbor-joining tree (Fig. 3), once several subclades with good support were separated from each other. All specimens of *C. odoratus* were collected in the vicinity of two dykes in the Dois Irmãos natural reserve. In turn, the *C. ligularis* individuals were found in various habitats, including the same as *C. odoratus*, and in different heavily perturbed open areas located nearby (Figs. 1, 3). The variability observed for *C. odoratus* may be related to higher levels of intraspecific polymorphism due to seedling recruitment, as previously reported for *C. papyrus* (Triest *et al*. 2014). On the other hand, the lower genetic differentiation observed in *C. ligularis* may be related to vegetative reproduction and/or a more efficient seed dispersion, which occurs by ornithochory, mirmechory, anemochory and mainly hydrochory (Linder & Rudall 2005), thus enhancing gene flow and reducing spatial aggregation among individuals (Triest *et al*. 2014). Moreover, we observed on the field that *C. odoratus* presents smaller clusters with more spherical rhizomes with lower density and a low number of clones, whereas *C. ligularis* has longer and thicker rhizomes with a higher number of clonal individuals. These different rates of clonal individuals between both species are probably related to the higher genetic diversity found for *C. odoratus*.

Karyotype analysis

Both species presented an intrapopulational variation in chromosome numbers, as it has been reported for other Cyperaceae genera (see Hipp 2007; Roalson 2008; Escudero *et al*. 2010). For *C. odoratus*, two chromosome numbers were observed: $2n = 72$ (in 67 % of the cells) and $2n = 76$ (in 33 % of the cells). On the other hand, *C. ligularis* showed a higher chromosome number variation, in which the diploid number ranged from $2n = 66$ to 88, with $2n = 82$ as the most frequent

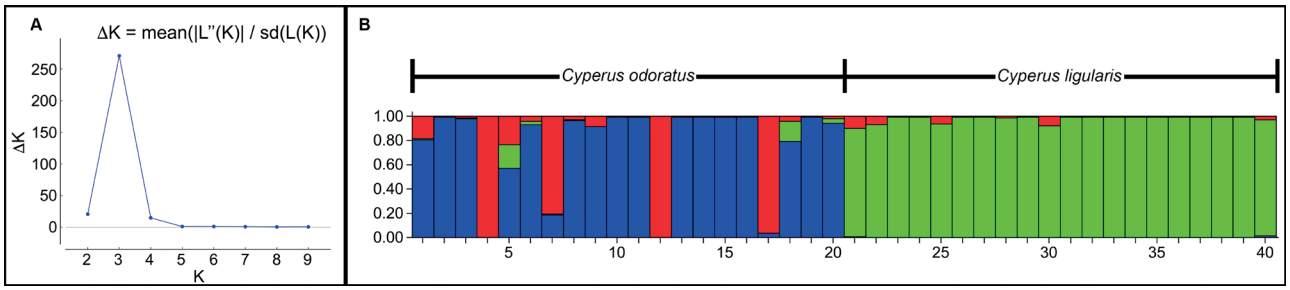

Figure 2. Bayesian clustering analysis based on Structure 2.3.3. **A.** Value of Delta K in accordance with the method described by Evanno *et al*. (2005). From the results obtained, 10 DAF primers were used, and 172 polymorphic bands were obtained for *Cyperus odoratus* and *C. ligularis*. Note that the peak occurred in K = 3. **B.** Ancestry of each individual in either of the two groups plotted by the software Structure version 2.3.4, using 10 DAF markers and 172 polymorphic bands for *C. odoratus* (individuals 1 – 20) and *C. ligularis* (individuals 21 – 40). Each vertical bar represents one of 40 individuals analyzed, and its genetic patrimony is noted through different colors (red, green and blue). Bar length is proportional to the ancestry values inferred in each group for each individual.

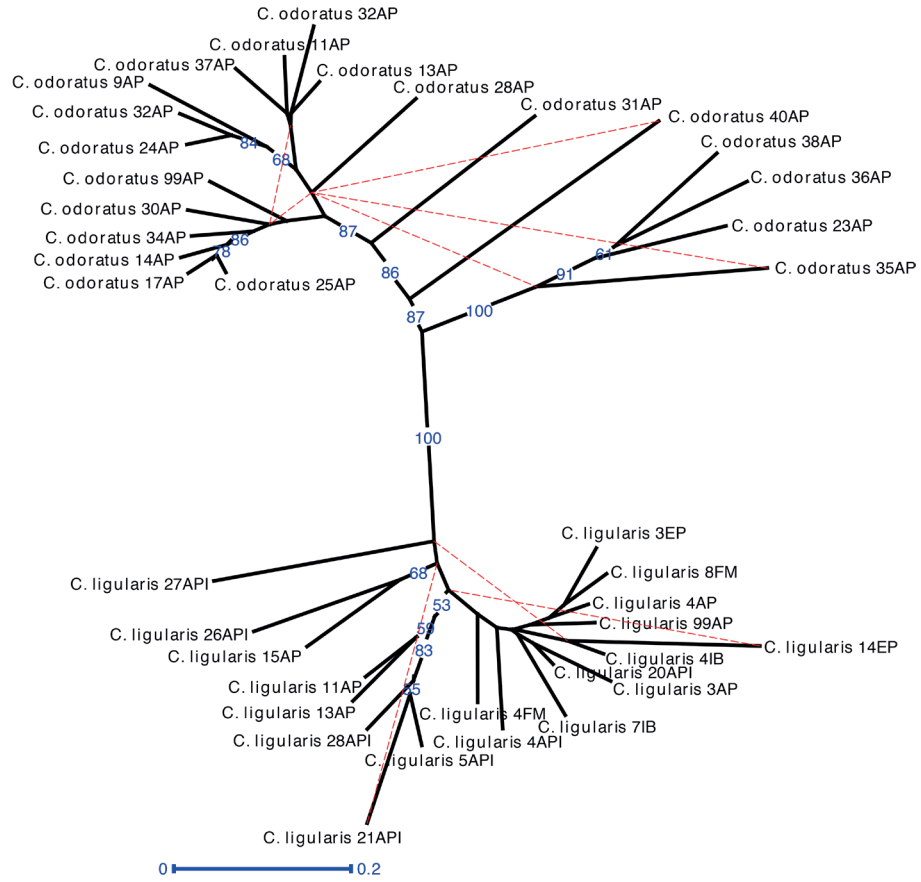

Figure 3. Neighbor-joining clustering analysis among 40 individuals of *Cyperus odoratus* and *C. ligularis* (Jaccard's coefficient and bootstrap of 2000 replicates). Collection plots: AP = Açude da Prata; API = Distrito de Apipucos; EP = Estrada dos Passarinhos; FM = Fábrica da Macaxeira; IB = Ilha do Bananal. Bootstrap values below 50 were not evidenced.

one (40 % of the cells) (Tab. S2, Fig. S1 in supplementary material). For both species, the CMA/DAPI banding always revealed two pairs of $CMA^+/DAPI^-$ signals (Fig. 4), which were associated with the two satellited chromosome pairs, also revealed by the silver nitrate impregnation (S Vasconcelos unpubl. res.).

Contrasting with the significant amount of published data on the karyological features of some genera of Cyperaceae, such as *Carex*, *Eleocharis* and *Rhynchospora* (Vanzela & Guerra 2000; Roalson 2008; Silva *et al*. 2010; Cabral *et al*. 2014), very little is known about the chromatin organization in *Cyperus* species. In fact, to the best of our knowledge, this is the first time that heterochromatic regions are evidenced in chromosomes of *Cyperus* species by means of fluorochrome banding. Consequently, it is hard to know if the coincident number of nucleolar organizer

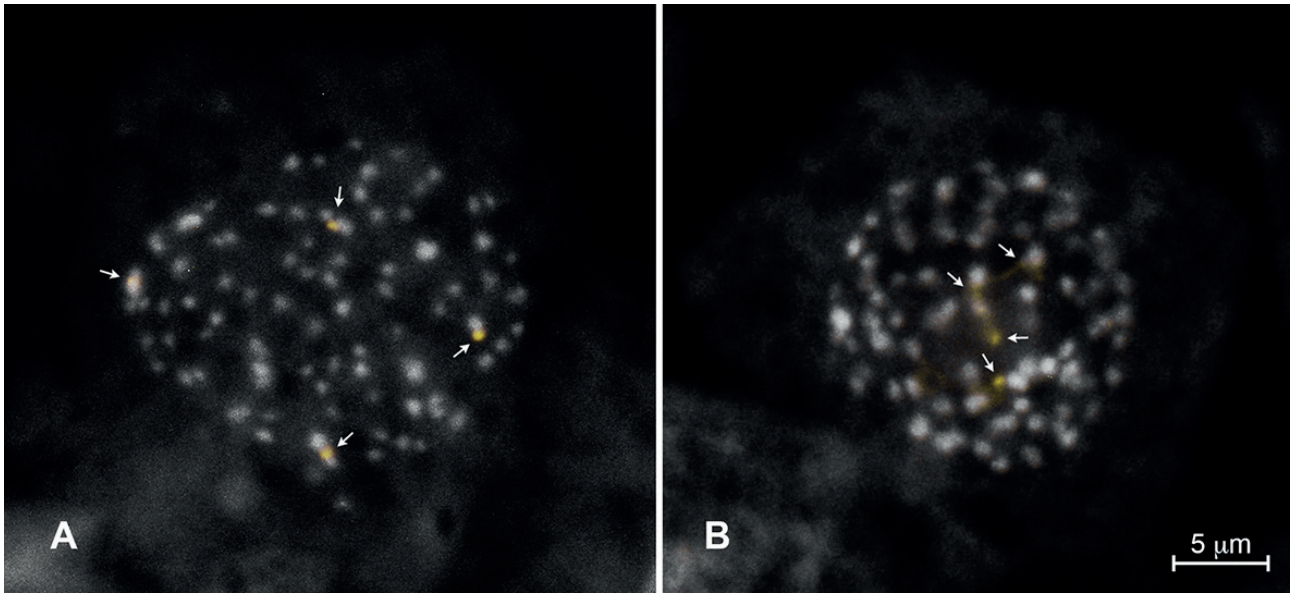

Figure 4. Mitotic metaphases of *Cyperus ligularis* and *C. odoratus* stained with CMA and DAPI. Cell of *C. ligularis* with 2*n* = 82. **A.** Cell of *C. odoratus* with 2*n* = 72 **B.** Arrows indicate satellite regions with bands $CMA^{+}/DAPI^{-}$.

regions (NORs), which were evidenced by both CMA/DAPI and silver nitrate staining, indicates some conserved feature within the genus, as reported for other groups of Cyperaceae (Silva *et al*. 2010). Thus, even sharing the same number of active NORs, the different ranges of chromosome number variation among sympatric individuals of *C. odoratus* and *C. ligularis* reinforces the assumption of a clear separation of the two species.

The high degree of chromosome number variation in Cyperaceae members is widely attributed to the occurrence of holokinetic chromosomes in all members of the family, as well as in Juncaceae, its sister group. Thus, the presence of diffuse kinetochores may facilitate a faster karyotypic evolution by means of chromosome fission/fusion and/or translocations (Escudero *et al*. 2010; Chung *et al*. 2011). As demonstrated for *Carex* species and herein observed in the sampled populations, it seems that there is no correlation between geographic range, chromosome number variation and genetic diversity in these species with holokinetic chromosomes (see Escudero *et al*. 2010; Chung *et al*. 2011).

Final considerations

The natural interspecific hybridization is not rare in Cyperaceae, where species of several genera as *Carex*, *Schoenoplectus* and *Isolepis* present species with hybrid origin (Pedersen *et al*. 2016; Yano *et al*. 2010; 2016). Although the morphology-based suggestion of hybridization between *C. odoratus* and *C. liguraris* by Luceño & Alves (1997), our molecular markers and cytogenetic analyses showed no evidence of hybridization.

Regarding the genetic diversity, *C. odoratus* surprisingly presents higher levels than *C. ligularis*, which is likely a result of a long period of evolution (Sheng *et al*. 2006) where the genetic diversity could be preserved by local vegetative regrowth of individuals. In the case of *C. ligularis*, the dispersal syndrome of the seeds, mainly by hydrochory and related with the colonization ability under various environmental conditions, seems to play a significant role in keeping the gene flow among individuals from different localities. However, the chromosome numbers showed a different picture, with *C. odoratus* less variable than *C. ligularis*, probably indicating a faster karyotypic evolution in the last species.

These results should be associated with additional field observations and specific analysis of reproductive biology, in order to enrich the knowledge concerning the dynamics and structure of the studied populations, as well as the role of these species in the successional processes and environmental restoration.

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