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Karyotype asymmetry: again, how to measure and what to measure?

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Abstract

One of the most popular, cheap and widely used approaches in comparative cytogenetics – especially by botanists – is that concerning intrachromosomal and interchromosomal karyotype asymmetry. Currently, there is no clear indication of which method, among the many different ones reported in literature, is the most adequate to infer karyotype asymmetry (especially intrachromosomal), above all in view of the criticisms recently moved to the most recent proposal published. This work addresses a critical review of the methods so far proposed for estimation of karyotype asymmetry, using both artificial and real chromosome datasets. It is shown once again how the concept karyotype of asymmetry is composed by two kinds of estimation: interchromosomal and intrachromosomal asymmetries. For the first one, the use of Coefficient of Variation of Chromosome Length, a powerful statistical parameter, is here confirmed. For the second one, the most appropriate parameter is the new Mean Centromeric Asymmetry, where Centromeric Asymmetry for each chromosome in a complement is easily obtained by calculating the difference of relative lengths of long arm and short arm. The Coefficient of Variation of Centromeric Index, strongly criticized in recent literature, is an additional karyological parameter, not properly connected with karyotype asymmetry. This shows definitively what and how to measure to correctly infer karyotype asymmetry, by proposing to couple two already known parameters in a new way. Hopefully, it will be the basic future reference for all those scientists dealing with cytotaxonomy.

Keywords

Artificial chromosome datasets, chromosomal heterogeneity, karyotype asymmetry, asymmetry indices, interchromosomal asymmetry, intrachromosomal asymmetry, karyological parameters, Stebbins classification

Introduction

Cytotaxonomy is a branch of cytogenetics, devoted to the comparative study of karyological features for systematic and evolutionary purposes (Siljak-Yakovlev and Peruzzi 2012). Today, a number of data can be obtained by chromosome studies: chromosome number, karyotype structure, karyotype asymmetry, chromosome banding, FISH, GISH and chromosome painting (Stace 2000, Levin 2002, Graphodatsky et al. 2011, Guerra 2012). Among them, one of the most popular, cheap and widely used approaches – especially by botanists – is that concerning karyotype asymmetry.

The concept of karyotype asymmetry, i.e. a karyotype marked by the predominance of chromosomes with terminal/subterminal centromeres (intrachromosomal asymmetry) and highly heterogeneous chromosome sizes (interchromosomal asymmetry), was developed for the first time by Levitsky (1931). Later, Stebbins (1971), in his masterpiece "*Chromosomal evolution in higher plants*", proposed a quali-quantitative method for the estimation of karyotype asymmetry in twelve categories, by taking into account four classes (from 1 to 4), defined according to the increasing proportion of chromosomes with arm ratio <2:1, to be combined with three classes (from A to C) defined according to the increasing ratio between largest and smallest chromosome in a complement.

Concerning interchromosomal asymmetry, which is due to heterogeneity among chromosome sizes in a complement, other researchers proposed quantitative estimation methods in the following years. This is the case of the Rec index (Greilhuber and Speta 1976, Venora et al. 2002), the A_2 index (Romero Zarco 1986), the R ratio (Siljak-Yakovlev 1996), the CV_{CL} (Lavania and Srivastava 1992, Watanabe et al. 1999, Paszko 2006). The latter, actually a Coefficient of Variation, is a statistically correct parameter and is able to capture even small variation among chromosome sizes in a complement. Hence, the estimation method for interchromosomal asymmetry does not need to be further discussed here.

More complex and debated is the quantitative estimation of the intrachromosomal asymmetry, which is due to centromere position. To address this issue, the first quantitative index proposed was the TF% of Huziwara (1962), soon followed by the AsK% of Arano (1963). Then, further proposals were AsI% of Arano and Saito (1980), Syi (Greilhuber and Speta 1976, Venora et al. 2002), A_1 (Romero Zarco 1986), CG (Lavania and Srivastava 1992), A (Watanabe et al. 1999), CV_{CI} (Paszko 2006). The latter, a Coefficient of Variation of Centromeric Index, was claimed by Paszko (2006) to be the only parameter with statistical foundation. However, her proposal was recently strongly criticized by Zuo and Yuan (2011), who evidenced that CV_{CI} is not able to capture and quantitatively express the original meaning of karyotype asymmetry (i.e. the prevalence of telocentric-subtelocentric chromosomes), but only to quantify the relative variation (heterogeneity) among centromere positions in a karyotype. Hence, the problem of a correct intrachromosomal asymmetry estimation is still open.

Finally, a few authors tried to combine the two kinds of asymmetry in a single index, such as Lavania and Srivastava (1992) with DI, Paszko (2006) with AI. However, both these indices were strongly criticized, by Paszko (2006) and Peruzzi et al. (2009)

respectively, and their use has to be definitely discouraged. The aim of this review is to critically analyze the proposed methods for estimating intrachromosomal asymmetry and to elaborate the proposal for a new suitable estimator which should be: 1) strictly quantitative, 2) statistically correct, 3) not a dispersion or variability index.

Which kind of basic measures were used – and differently combined – for Intrachromosomal Asymmetry estimation?

Fundamentally, the basic measures, used in every method proposed so far, are those concerning the length of long (L) and short arm (S) of each chromosome in a complement. All the karyotypes where these measures are not applicable (for instance those with holocentric chromosomes or those with very small chromosomes, 1 μm or less), are not suitable for the estimation of intrachromosomal asymmetry at all. For all the others (the majority), typically $L \geq S \geq 0$ and $L \geq S$. The variation extremes are $S = L$ (i.e. chromosomes with centromere perfectly median) and $S = 0$ (i.e. chromosomes with centromere perfectly terminal). These two variables were combined by researchers in various ways:

L/S also called ***arm ratio*** **(r)**, it was used for instance in the widely known chromosome nomenclature proposed by Levan et al. (1964). Its values can range from **1** (if S = L) to **+∞** (the limit for S = 0).

S/L first proposed by Battaglia (1955), it is reciprocal to the arm ratio. Its values can range from **1** (if S = L) to **0** (if S = 0). It is fundamentally used also in **Syi** = (Mean S length / Mean L length) × 100 (Greilhuber and Speta 1976, Venora et al. 2002).

S/(L+S) also called ***centromeric index***, it is the proportion of short arm respect with the whole chromosome. Its values can range from **0.5** (if S = L) to **0** (if S = 0). It is fundamentally used in **TF%** = Total length of S in a chromosome set / Total length of a chromosome set × 100 (Huziwara 1962), **CG** = Median S length / Median (L+S) length × 100 (Lavania and Srivastava 1992), and $\mathbf{CV_{CI}}$ (Paszko 2006).

L/(L+S) it is the proportion of long arm respect with the whole chromosome, being complementary to the centromeric index. Indeed, [L/(L+S)] + [S/(L+S)] = 1. Its values can range from **0.5** (if S = L) to **1** (if S = 0). It is fundamentally used in **AsK%** = Total length of L in a chromosome set / Total length of a chromosome set × 100 (Arano 1963) and the identical **AsI%** (Arano and Saito 1980).

(L–S)/L it was conceived in order to be complementary to S/L, indeed [(L–S)/L]+S/L = 1. Its values can range from **0** (if S = L) to **1** (if S = 0). It is used in $\mathbf{A_1}$ = 1 – Mean S/L (Romero Zarco 1986).

(L–S)/(L+S) it is the difference between the two (complementary) proportions L/(L+S) and S/(L+S). Hence, its values can range from **0** (if S = L) to **1**

(if S = 0). It is used in **A** = Mean (L–S)/(L+S) (Watanabe et al. 1999). *Please note that it can be expressed also as 2L/(L+S) – 1 or as 1 – 2S/(L+S).*

Given that L/(L+S) and S/(L+S) are the only parameters which are formally correct on descriptive statistical grounds (they are both proportions, or relative lengths), and given their peculiar complementary relationships, the only parameter well suited to capture the mean intrachromosomal asymmetry in a karyotype is that proposed by Watanabe et al. (1999). It is noteworthy that these authors already stressed that their method is preferable respect with others "*because it usually follows a normal distribution*". Indeed, given an artificial dataset of chromosomes with normal distribution (mean = median), only the estimators L/(L+S), S/(L+S) and their difference (L–S)/(L+S) are able to correctly describe these features (Table 1). However, it also must be noted that all the other karyotype intrachromosomal asymmetry estimators proposed in literature (Syi, TF%, CG, AsK%, A_1), albeit not statistically correct, are highly correlated with A, with values typically above r = |0.9|, p < 0.01 (Paszko 2006, Peruzzi et al. 2009).

How to compare Karyotype Asymmetry among individuals, populations, species etc.?

Let us return to karyotype asymmetry as a whole, with its two parts: interchromosomal and intrachromosomal. Concerning the measure of interchromosomal asymmetry, as explained above, the main point is to measure how much the chromosome lengths of a complement are different each other, and CV_{CL} (Paszko 2006) is perfectly suited for it. As all coefficients of variation, it is a ratio between standard deviation and mean of a sample (i.e. a dispersion index) × 100 (Sokal and Rohlf 1981). Typically, this parameter ranges from 0 (no variation) to 100 or more (in those cases of exceptionally heterogeneous samples, where standard deviation can be higher than the mean).

Table 1. Comparison of different estimators of intrachromosomal asymmetry on a set of 11 artificial chromosomes with gradually increasing asymmetry, from perfectly median (on the left) to perfectly terminal (on the right) centromeres. Also the mean values are reported in the last column on the right. L/S was excluded because no real value is obtained when S = 0.

	chromosome											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**mean**
S (μm)	10	9	8	7	6	**5**	4	3	2	1	0	**5**
L (μm)	10	11	12	13	14	**15**	16	17	18	19	20	**15**
S/L	1.00	0.82	0.67	0.54	0.43	0.33	0.25	0.18	0.11	0.05	0.00	0.40
S/(L+S)	0.50	0.45	0.40	0.35	0.30	0.25	0.20	0.15	0.10	0.05	0.00	**0.25**
L/(L+S)	0.50	0.55	0.60	0.65	0.70	0.75	0.80	0.85	0.90	0.95	1.00	**0.75**
(L-S)/L	0.00	0.18	0.33	0.46	0.57	0.67	0.75	0.82	0.89	0.95	1.00	0.60
(L-S)/(L+S)	0.00	0.10	0.20	0.30	0.40	**0.50**	0.60	0.70	0.80	0.90	1.00	**0.50**

Concerning the measure of intrachromosomal asymmetry, CV_{CI} should not be used for the reasons explained above. Indeed, it is actually a measure of intrachromosomal heterogeneity, which does not necessarily means asymmetry in the original sense given by Levitsky (1931) and Stebbins (1971). Among others, as shown above, the statistically best suited parameter is A (Watanabe et al. 1999), ranging from 0 (perfectly symmetric) to 1 (perfectly asymmetric).

Since the two kinds of asymmetry express different concepts, it is not desirable to combine them in a single value. On the contrary, as argued for the first time by Romero Zarco (1986) and then by Peruzzi et al. (2009), the best way in representing karyotype asymmetry relationships among organisms is by means of bidimensional scatter plots, where the two asymmetry estimators are put in the x and y axes and points represent each sample. Up to the present day, this was done with the couples of parameters A_1 and A_2 (Romero Zarco 1986) or CV_{CI} and CV_{CL} (noteworthy, $CV_{CL} = A_2 \times 100$) (Paszko 2006; Peruzzi et al. 2009).

The present proposal is to couple CV_{CL} with a new parameter called **M_{CA} (Mean Centromeric Asymmetry)**, where Centromeric Asymmetry of a single chromosome is given by the formula (L-S)/(L+S). Accordingly, $M_{CA} = A \times 100$. Generally, CV_{CI} is not correlated with M_{CA} (e.g. in small dataset of *Calamagrostis* Adanson, 1753 used by Paszko 2006), so that it could be used sometimes as an optional third parameter to reveal karyotype relationships among organisms, besides asymmetry *sensu stricto*. This could be useful especially when chromosome size variation is negligible.

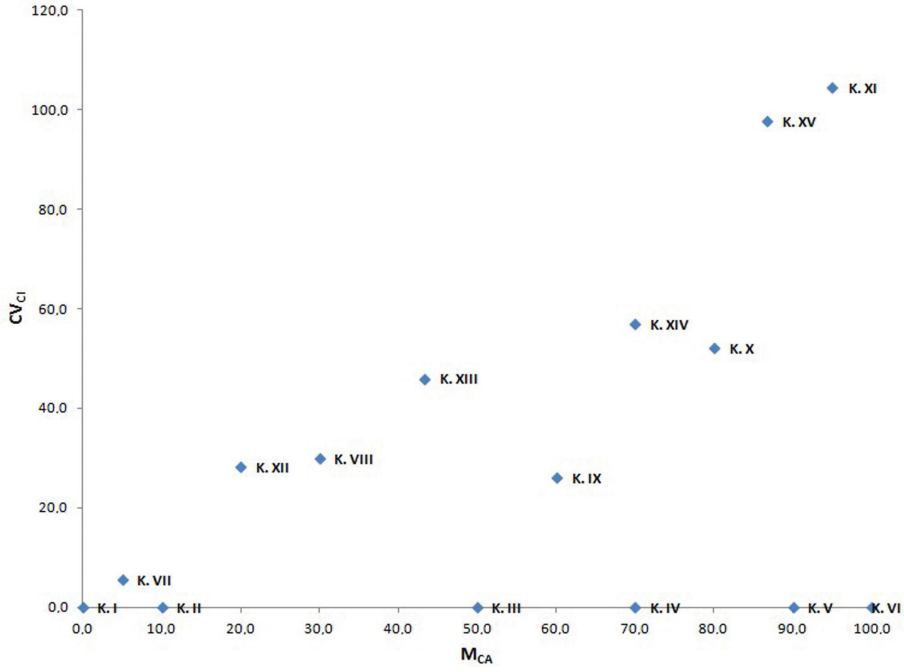

Figure 1. Scatter plot of the fifteen artificial karyotypes reported in Table 2 against M_{CA} (x axis) and CV_{CI} (y axis).

Table 2. Karyomorphometric features of a dataset with fifteen artificial karyotypes, all with the chromosomes of the same length (no chromosome size variation).

	chromosome																							
	1		2		3		4		5		6		7		8		9		10		11		12	
karyotype	L	S	L	S	L	S	L	S	L	S	L	S	L	S	L	S	L	S	L	S	L	S	L	S
karyotype I	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
karyotype II	5.5	4.5	5.5	4.5	5.5	4.5	5.5	4.5	5.5	4.5	5.5	4.5	5.5	4.5	5.5	4.5	5.5	4.5	5.5	4.5	5.5	4.5	5.5	4.5
karyotype III	7.5	2.5	7.5	2.5	7.5	2.5	7.5	2.5	7.5	2.5	7.5	2.5	7.5	2.5	7.5	2.5	7.5	2.5	7.5	2.5	7.5	2.5	7.5	2.5
karyotype IV	8.5	1.5	8.5	1.5	8.5	1.5	8.5	1.5	8.5	1.5	8.5	1.5	8.5	1.5	8.5	1.5	8.5	1.5	8.5	1.5	8.5	1.5	8.5	1.5
karyotype V	9.5	0.5	9.5	0.5	9.5	0.5	9.5	0.5	9.5	0.5	9.5	0.5	9.5	0.5	9.5	0.5	9.5	0.5	9.5	0.5	9.5	0.5	9.5	0.5
karyotype VI	10	0	10	0	10	0	10	0	10	0	10	0	10	0	10	0	10	0	10	0	10	0	10	0
karyotype VII	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.5	4.5	5.5	4.5	5.5	4.5	5.5	4.5	5.5	4.5	5.5	4.5
karyotype VIII	5.5	4.5	5.5	4.5	5.5	4.5	5.5	4.5	5.5	4.5	5.5	4.5	7.5	2.5	7.5	2.5	7.5	2.5	7.5	2.5	7.5	2.5	7.5	2.5
karyotype IX	7.5	2.5	7.5	2.5	7.5	2.5	7.5	2.5	7.5	2.5	7.5	2.5	8.5	1.5	8.5	1.5	8.5	1.5	8.5	1.5	8.5	1.5	8.5	1.5
karyotype X	8.5	1.5	8.5	1.5	8.5	1.5	8.5	1.5	8.5	1.5	8.5	1.5	9.5	0.5	9.5	0.5	9.5	0.5	9.5	0.5	9.5	0.5	9.5	0.5
karyotype XI	9.5	0.5	9.5	0.5	9.5	0.5	9.5	0.5	9.5	0.5	9.5	0.5	10	0	10	0	10	0	10	0	10	0	10	0
karyotype XII	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.5	4.5	5.5	4.5	5.5	4.5	5.5	4.5	7.5	2.5	7.5	2.5	7.5	2.5	7.5	2.5
karyotype XIII	5.5	4.5	5.5	4.5	5.5	4.5	5.5	4.5	7.5	2.5	7.5	2.5	7.5	2.5	7.5	2.5	8.5	1.5	8.5	1.5	8.5	1.5	8.5	1.5
karyotype XIV	7.5	2.5	7.5	2.5	7.5	2.5	7.5	2.5	8.5	1.5	8.5	1.5	8.5	1.5	8.5	1.5	9.5	0.5	9.5	0.5	9.5	0.5	9.5	0.5
karyotype XV	8.5	1.5	8.5	1.5	8.5	1.5	8.5	1.5	9.5	0.5	9.5	0.5	9.5	0.5	9.5	0.5	10	0	10	0	10	0	10	0

For instance, in an artificial karyotypes dataset with no chromosome size variation (Table 2), it is once again evident how samples, even with the most different intrachromosomal asymmetries (M_{CA}), could not be discriminated by CV_{CL} if there is not variation in chromosome size. Conversely, samples with almost identical intrachromosomal asymmetry can reveal their different karyotype structure following use of the CV_{CI} (compare karyotypes III and XIII, or IV and XIV, in Figure 1). In some special case, if CV_{CI} results positively correlated with M_{CA}, this former additional karyological parameter is not useful at all and may be omitted. This is the case of the large Liliaceae dataset used by Peruzzi et al. (2009), where the correlation among CV_{CI} and M_{CA} is $r = 0.792$ ($p < 0.01$). As can be seen in Figure 2, the three tribes of subfamily Lilioideae show a clear tendency to have karyotypes distinct on asymmetry grounds: tribe Medeoleae, with relatively low intrachromosomal (M_{CA}) and interchromosomal asymmetry (CV_{CL}), tribe Tulipeae, with higher interchromosomal asymmetry, and tribe Lilieae, with higher intrachromosomal asymmetry. Almost identical results were indeed obtained by Peruzzi et al. (2009) by using the, now "old-fashioned", couple CV_{CI} and CV_{CL}. Finally, it is also important to remember here, once again, that a symmetric karyotype does not necessarily implies "primitivity", as assumed by earlier students (see, for instance, Siljak-Yakovlev 1996 for the concept of "secondary symmetry"). As for other cytotaxonomic features, once

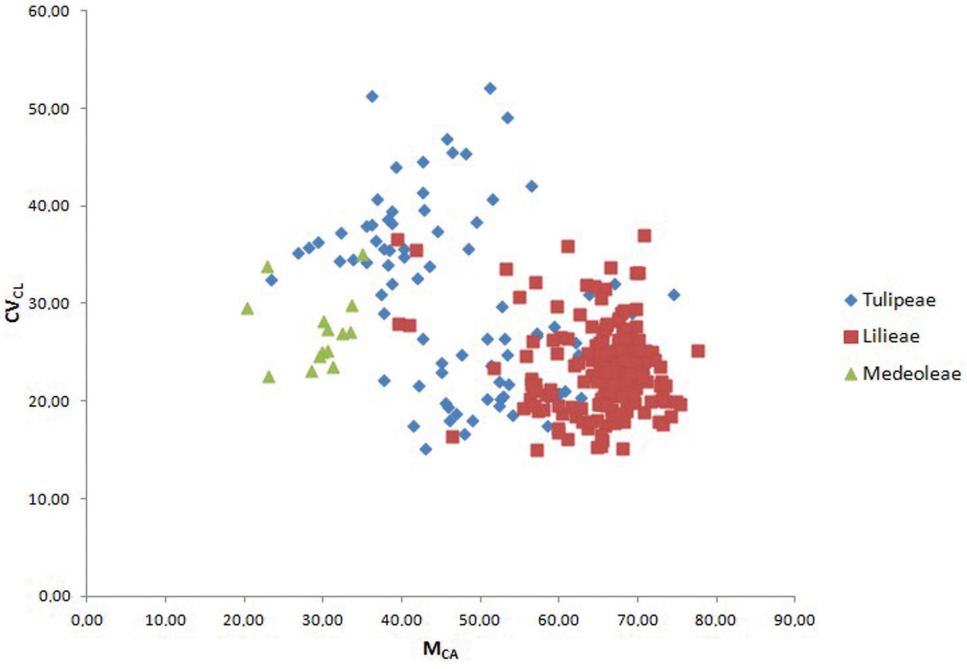

Figure 2. Scatter plot of samples from the three tribes Medeoleae, Tulipeae and Lilieae against M_{CA} (x axis) and CV_{CL} (y axis). Data derived from the dataset published by Peruzzi et al. (2009).

karyological relationships between taxa are demonstrated, it is also important to have some independent source of information in order to infer the direction of changes (Siljak-Yakovlev and Peruzzi 2012).

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Chromosomal homology of *Uraeotyphlus oxyurus* group of species (Amphibia, Gymnophiona, Ichthyophiidae)

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Abstract

Uraeotyphlus oxyurus (Dumeril et Bibron, 1841), *U. interruptus* Pillai et Ravichandran, 1999, *U. narayani* Seshachar, 1939 and *U. menoni* Annandale, 1913 were cytogenetically analysed following conventional and differential staining techniques. These species show similar karyotypes with 2n=36 (FN=58). There were no traces of species-specific features in regard to C-banding and NOR staining. The comparative study of karyotypes shows chromosomal homologies among the four species. Chromosomal data seem to support the concept that two species groups exist in the genus *Uraeotyphlus*.

Keywords

Uraeotyphlus oxyurus species group, karyotypes, chromosomal homology

Introduction

The genus *Uraeotyphlus* Peters, 1879 is endemic to the Western Ghats region of peninsular India and constitutes one of the three genera within the family Ichthyophiidae Taylor, 1968 along with *Caudacaecilia* Taylor, 1968 and *Ichthyophis* Fitzinger, 1826 (Wilkinson et al. 2011). Its taxonomy had been uncertain till the publication of Nussbaum and Wilkinson (1989) which gave this group a family level status among the existing caecilians of India. However, after a lapse of a decade or so, this prevailing situation seemed to have recovered moderately and is sufficient in redefining interrelationships among other families of caecilians based on morphological and molecular evidence (Wilkinson and Nussbaum 1996, 2006, Frost et al. 2006, Gower and Wilkinson 2007, Roelants et al. 2007, Gower et al. 2008, Zhang and Wake 2009, Wilkinson et al. 2011).

On the basis of morphological features such as cylindrical body, annulation and of the presence or absence of phallodeum, and of limited molecular evidence, Gower and Wilkinson (2007) partitioned this supposedly monophyletic genus *Uraeotyphlus* into two species groups: *U. oxyurus* species complex and *U. malabaricus* species group. Consequent upon this arrangement, *Uraeotyphlus oxyurus* (Dumeril & Bibron, 1841), *U. interreptus* Pillai & Ravichandran, 1999, *U. narayani* Seshachar, 1939 and *U. menoni* Annandale, 1913 were grouped as derived species, while of *U. gansi* Gower, Rajendran, Nussbaum & Wilkinson, 2008, *U. oommeni* Gower & Wilkinson, 2007 and *U. malabaricus* (Beddome, 1870) were considered as primitive ones (Gower et al. 2008).

Earlier, Seshachar (1939) presented the male meiotic chromosomal complement of *U. narayani* with the dipoloid number of 36 and gave detailed descriptions based on chromosomal morphs observed such as V-shaped, rods and dots in the complement. Elayidom et al. (1963) have described the somatic and meiotic chromosomal complement of *U. menoni* as consisting of diploid number of 36 for the species, but neither study presented karyotypic characteristics. Venkatachalaiah and Venu (2002) gave a detailed karyotypic characteristic of what was then mistakenly thought to be *Ichthyophis malabarensis* Taylor, 1960 (2n=36, FN= 60) but which was subsequently found to be *U.* prope *interruptus* of Ichthyophiidae. With this surge of interest to elucidate the phylogeny and evolution, Venu et al. (2011) have presented the chromosomes of a member of the *U. malabaricus* species group, *U. gansi,* bearing 2n=42, FN=58, highest diploid number known thus far for a member of the genus *Uraeotyphlus* of the family Ichthyophiidae.

In this study, we present the karyotypes of *U. oxyuryus* and *U. interruptus* and the results of reanalysis of chromosomes of *U. narayani* and *U. menoni* with a view to providing new insights into the intragenus relationships within the genus *Uraeotyphlus*.

Material and methods

Specimens of both sexes, collected from different regions the Western Ghats (Table 1) a few days before the experiment, were kept in glass aquaria under suitable conditions. After *in vivo* colchicine treatment, chromosome preparations were obtained from the liver, the gut epithelium and the testis. Cell suspensions, hypotonic treatment and fixation of cells were performed as described earlier (Venkatachalaiah and Venu 2002, Venu et al. 2011). Chromosome number and standard karyotype (in respect of somatic metaphase and meiotic pachytene) morphology were determined by conventional Giemsa staining technique. Chromosome nomenclature was followed as proposed earlier by Levan et al. (1964) but adopted for the present situation as described earlier (Venkatachalaiah and Venu 2002).

Conventional C-banding was performed according to Sumner (1972) using $Ba(OH)_2$ at 60^0C followed by staining in dilute Giemsa solution, with modifications in alkaline treatment.

Location of nucleolus organizer regions (NORs) was performed by applying the one-step silver nitrate method of Goodpasture and Bloom (1975).

Table 1. Details of collection of *Uraeotyphlus interruptus*, *Uraeotyphlus narayani*, *Uraeotyphlus menoni* and *Uraeotyphlus oxyurus*.

Species	Locality	Habitat	Voucher number	No. of animals used	Geographical coordinates
Uraeotyphlus interruptus	Gudalur, Nilgiris (Dt), Tamil Nadu, India	Mixed plantations of tea, banana, pepper, orange, coffee	BUB114, 103 BUB105, 111	2 males 2 females	11°30'0"N 76°30' 0"E
Uraeotyphlus narayani	Changanssery, Kottayam (Dt), Kerala, India	Backyard garden with banana plantation	BUB101, 115 BUB109, 116	2 males 2 females	9°28'00"N 76°33'00"E
Uraeotyphlus menoni	Mattathur, Thrissur (Dt), Kerala, India	Backyard garden with banana plantation	BUB107, 113 BUB106, 102	2 males 2 females	10°22'45"N 76°19'15"E
Uraeotyphlus oxyurus	Agali, Palakkad (Dt), Kerala, India	Cultivated agricultural land with banana and coconut plantation	BUB104, 112 BUB108, 110	2 males 2 females	11°5'0"N 76°35'0"E

Results

Karyotypes of *Uraeotyphlus oxyurus* and *U. interruptus*

The karyotypes of *U. oxyurus* (Fig. 1) and *U. interruptus* (Fig. 2) revealed a diploid chromosomal complement consisting of 2n=36, FN=58.

The somatic metaphase chromosomes in the karyotype could be divided into four groups, A, B, C and D, based on the decreasing order of total length and position of centromere of each chromosome. The first group (A) includes two pairs (1–2) of large metacentrics and one pair (3) of submetacentrics, while the B group consisted of three pairs (4–6) of medium sized metacentrics in which the pair four was slightly longer than other two pairs. The third group (C) included 5 pairs (7–11) of smaller submetacentrics, all in decreasing order of their total length. The fourth group (D) included mostly acrocentric (12–18) pairs.

Similar karyotypes were obtained for the species *U. narayani* (Fig. 3) and *U. menoni* (Fig. 4).

No detectable sex chromosome pair was observed in either sex in the metaphase chromosomal complement and karyotypes of *U. oxyurus*, *U. interruptus*, *U. narayani* and *U. menoni*.

Meiosis

The meiotic chromosomes prepared from male individuals of *U. oxyurus* revealed a good number of pachytene (Fig. 5), diplotene (Fig. 6), diakinetic and second meiotic metaphase configurations.

Pachytene chromosome karyotype constructed as per the somatic metaphase chromosome karyotype revealed eighteen pachytene bivalents corresponding the eighteen pairs of somatic chromosomes.

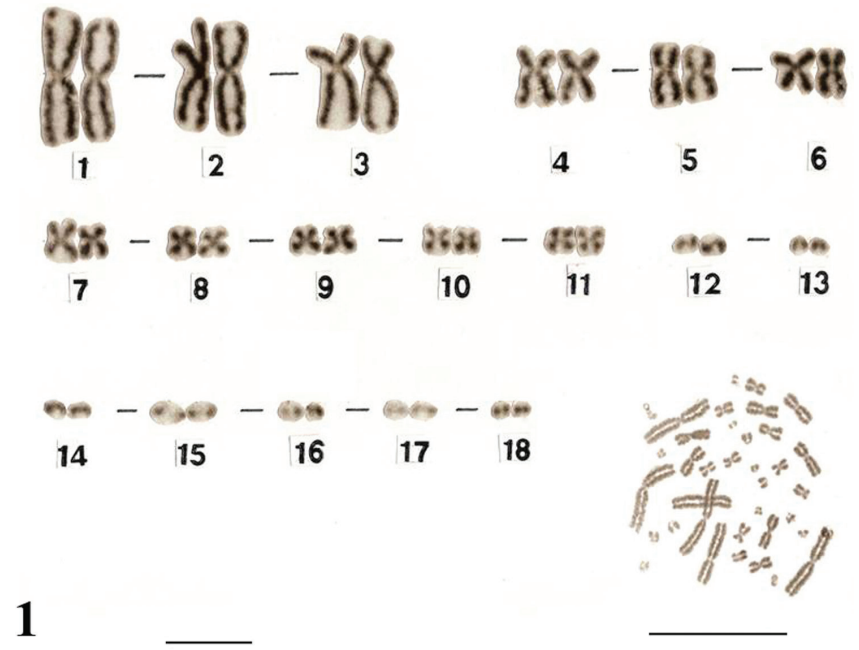

Figure 1. Giemsa stained male karyotype and female metaphase complement of *Uraeotyphlus oxyurus*. Bar = 10 μm.

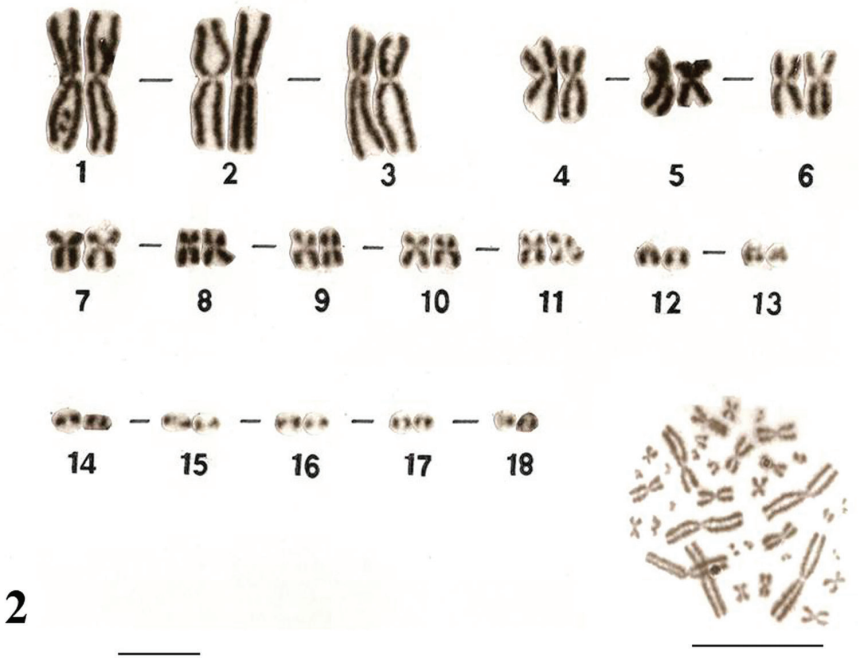

Figure 2. Giemsa stained male karyotype and female metaphase complement of *Uraeotyphlus interruptus*. Bar = 10 μm.

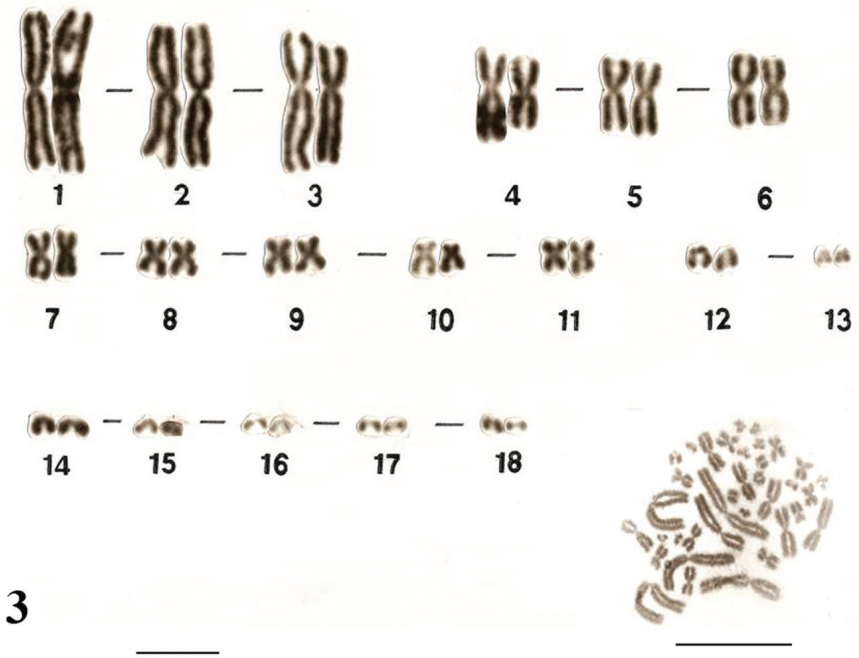

Figure 3. Giemsa stained male karyotype and female metaphase complement of *Uraeotyphlus narayani*. Bar = 10 μm.

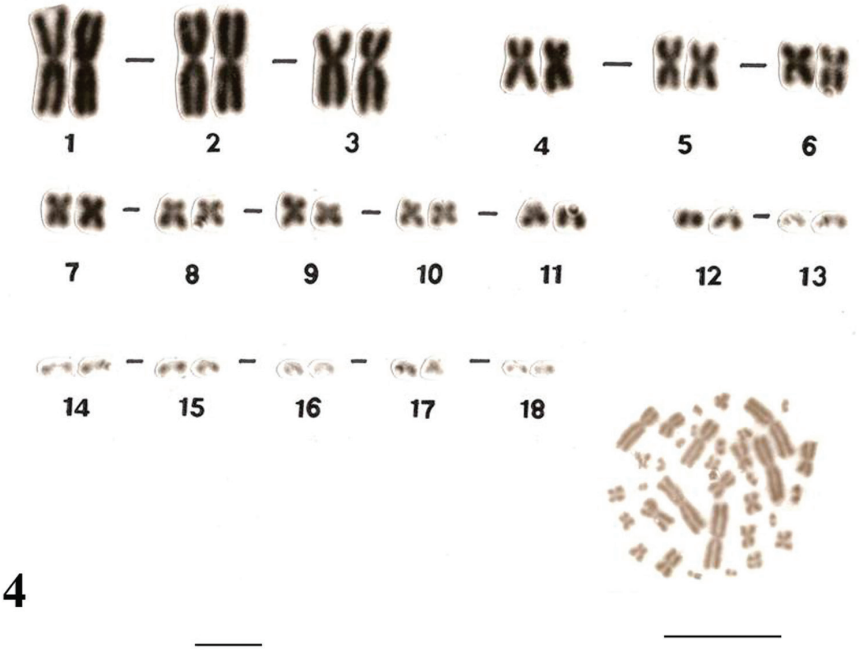

Figure 4. Giemsa stained male karyotype and female metaphase complement of *Uraeotyphlus menoni*. Bar = 10 μm.

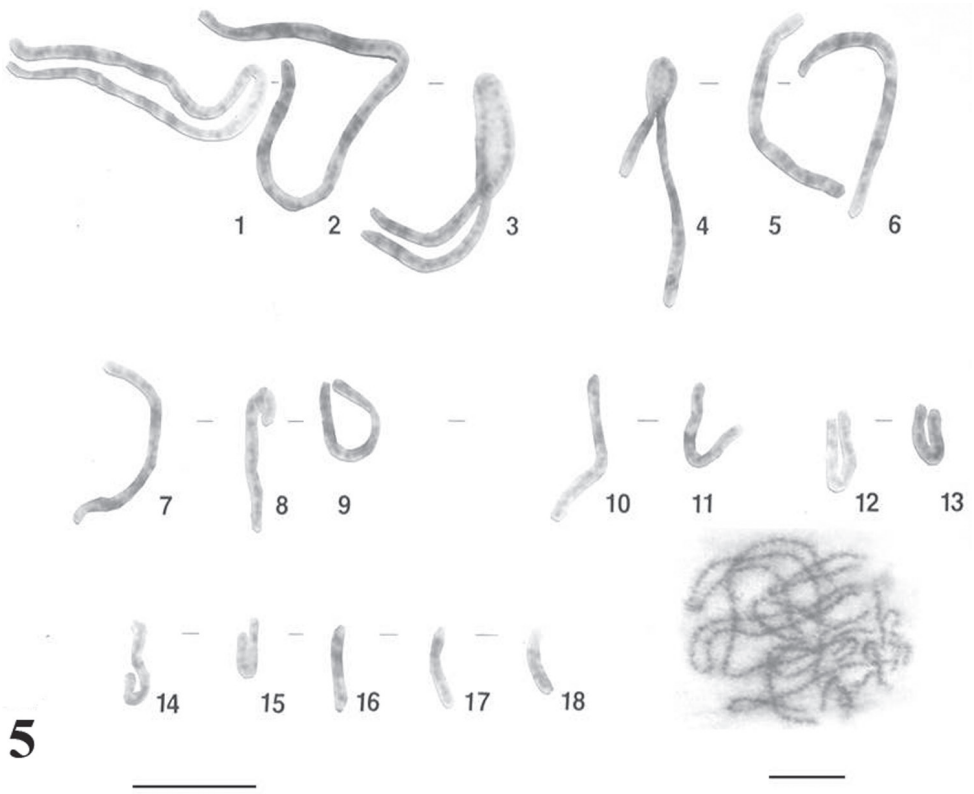

Figure 5. Pachytene karyotype and complement of *Uraeotyphlus oxyurus*. Bar = 10 μm.

The diplotene complement allowed the counting of eighteen individually identifiable bivalents. In each diplotene complement, the longer ones carried 4-6 chiasmata, whereas the smaller acrocentrics consisting of at least one chiasma.

Similar results were obtained for the other three species, *U. interruptus*, *U. narayani* and *U. menoni*.

C – Staining

The *U. oxyurus* karyotype is characterized by discernible but faintly stained centromeric C-bands in the metacentric and submetacentric chromosomes, while the acrocentrics have very prominent C-bands at the centromeric region cumulatively highlighting both the centromeric regions and the proximal portions of each short arms of each chromosome (Fig. 7). In comparison with the C-staining characteristics of *U. oxyurus,* typical C-bands were observed in the other three species karyotypes.

Figure 6. Diplotene complement of *Uraeotyphlus oxyurus*. Bar = 10 μm.

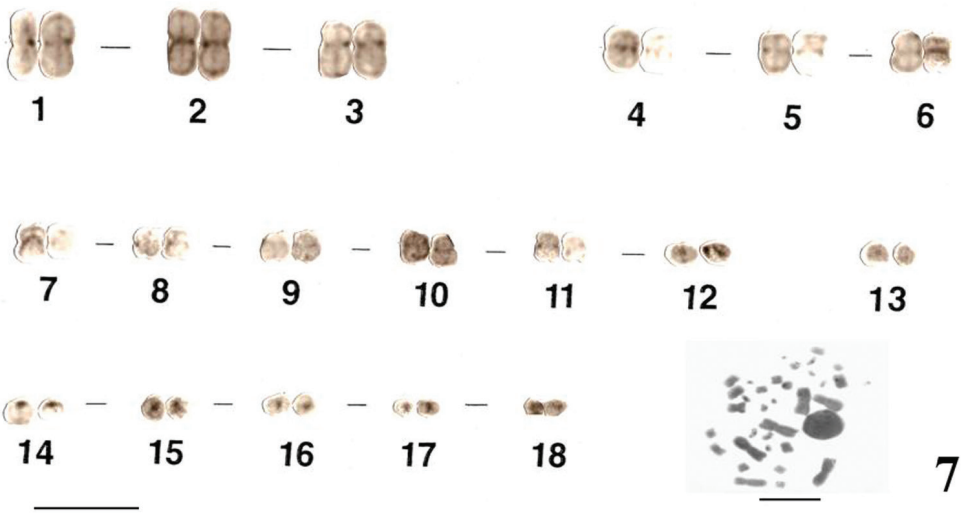

Figure 7. C-stained male karyotype and female metaphase complement of *Uraeotyphlus oxyurus*. Bar = 10 μm.

Ag-NOR – Staining

Silver nitrate staining showed that in *U. oxyurus,* Nucleolus Organizer Regions (NORs) are confined to chrormosomal pair 9 in the complement but were not consistently demonstrated in each and every cell. Whereas, interphase nuclei demonstrated 1 to 2 (or sometimes 3) numbers of silver nitrate (2–3) aggregates (Fig. 8). This situation is perhaps an indication of limited proportionality of rDNA sites that could not be elicited cytologically and those of transcriptionally silent NORs did not form any discrete Ag-NORs sites at metaphase chromosomes.

Similar kind of results could be drawn for the other three species, *U. interruptus*, *U. narayani* and *U. menoni*.

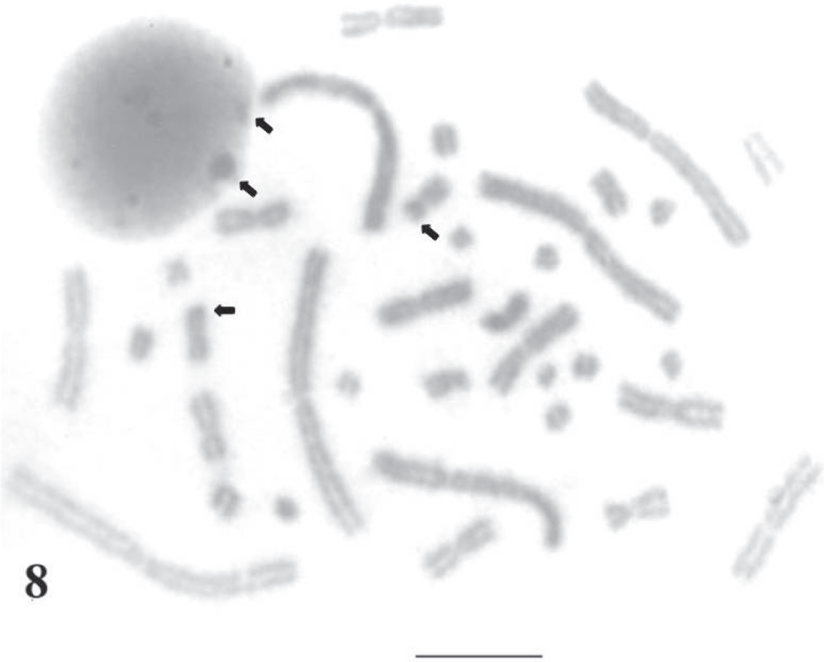

Figure 8. Silver-stained interphase nucleus and mitotic metaphase complement of *Uraeotyphlus oxyurus*. Bar = 10 μm.

Discussion

Emphasizing on systematics of those Indian endemic uraeotyphlids, the morphological attributes as elicited by Gower and Wilkinson (2007) and Gower et al. (2008) are in congruence with the results of cytogenetic data that were available for determining their primacy and prevalence of bimodal karyotypic characteristics.

In the present study, the karyotype carrying a diploid number of 36 (2n=36) chromosomes was found consistently identical in each of the four species belonging to *U. oxyurus*

species complex. The cytogenetic data converged on chromosome morphology indicate that the genus *Uraeotyphlus* is relatively well conserved with most chromosomal pairs classified as meta- and submetacentrics in size and shape. This observation, especially of all the four *oxyurus* type karyotypes having a homologous situation reveals a closer phylogenetic relationship. This, in turn, supports consideration for a monophyletic origin.

The same situation cannot be considered as applying to those species belonging to *U. malabaricus* group. Until now, none of these species karyotypes were known except a publication citing a variable karyotype for the species belonging to the genus, *Uraeotyphlus*, *U. gansi*. Venu et al. (2011) have described the karyotype of *U. gansi* bearing a different basic chromosomal number (2n=42) and morphology. A major attribute of *U. gansi* karyotype is that it is similar to any of the known karyotypes of the species belonging to *Ichthyophis* and *Caudacaecilia* of the family Ichthyophiidae (Venkatachalaiah and Venu 2002, Matsui et al. 2006, Venu 2008, Venu et al. 2011).

This karyolgical description lends support to Gower and Wilkinson's (2007) contention that it is possible to draw conclusions as to the karyological affinities between the two taxa through a comparison of the primitive uraeotyphlid karyotype with that of ichthyophiid karyotypes. The karyogrammic and morphometric data on the karyotype of *U. gansi* and that of representative karyotype of *Ichthyophis* and *Caudacaecilia* (Nussbaum 1991; Venkatachalaiah and Venu 2002; Matsui et al. 2006; Venu 2008) lend support to the concept of closer phylogenetic affinity between the two taxa within the family (Wilkinson et al. 2011, Venu et al. 2011).

The four species karyotypes (2n=36) may be considered as derived ones from that of *U. gansi* karyotype (2n=42), which serves as a modal karyotype for the *U. malabaricus* group species which could be considered a basal one among uraeotyphlids. Besides, based on the pronounced chromosomal homogeneity among the said species groups' karyotypic specificities, this may be considered as of cytogenetic importance while placing emphasis on species differentiation within the genus. Uraeotyphlid chromosome complements belonging to family Ichthyophiidae seem to present bimodal diploid numbers: 2n=42 chromosomes in the basal type *U. malabaricus* group and 2n=36 chromosomes in the case of derived species belonging to *U. oxyurus* species complex.

Presently, chromosomal data on ichthyophiid taxa indicate that they are all well-conserved among the species whose karyotypes are known, bearing identical chromosomal set in each case (Venkatachalaiah and Venu 2002; Venu 2008). The characteristics of the four *U. oxyurus* karyotypes and of *U. gansi* karyotype are unique in possessing most chromosomal pairs as conserved. The centromeric position of the lowerset of chromosomes in the karyotype makes all the more significant for consideration of their karyotypic specificities.

During the course of drawing karyological relationships prevailing between the two taxa (for e.g., ichthyophiids and uraeotyphlids) it is possible to infer that most submeta and metacentrics are conserved to a maximum extent and only acrocentrics mayhave paved the way for chromosome speciation events.

There are instances that exhibit very little variation in their karyological features. Marked karyological homogeneity seems prevalent in many taxa of cyprinid fishes

(Caputo et al. 2003, Rabova et al. 2003, Mesquita et al. 2008) and in some salamanders (Sessions 2008) and anuran amphibians (Kasahara et al. 2003, Aguiar et al. 2004, Rodrigues et al. 2011) and evidently in cryptodire reptilians (Olmo 2005, Castiglia et al. 2009). Karyological uniformity as demonstrated by some of these studies (including the present report) seems to point towards eliciting closer affiliation in their respective lineages. Moreover, this kind of situation in the context of phylogenetic assessment reflects upon their initial stages of evolutionary consequences.

Any attempt on comparison of karyotypes from the present study to that of other representative karyotypes for *U. malabaricus* type (although only one species karyotype is available) makes clear the occurrence of a succession of chromosomal rearrangements, mainly through pericentric inversion and / or fusion. This appears necessary in order to create the karyotype found in described karyotype from those of ancestral ones. This sequence of events could account for the appearance of reduction in basic chromosome numbers from 2n=42 chromosome to 2n=36 chromosomes (Venkatachalaiah and Venu 2002).

The C-banding profile and NOR localization seems to be a homologous feature in *oxyurus* uraeotyphlids. C-banding pattern was found identical in the four species of *oxyurus* group within the genus. The variation illustrated by the two species group (viz., *oxyurus* and *malabaricus*) in which there is an enormous interspecific variation was evident in the distribution and amount of heterochromatin (Venu 2008; Venu et al. 2011). The NOR location is a conserved characteristic within the *oxyurus* group species. This view is in accordance with that of Schmid et al (1990) opinion that in closely related species, the NORs are always almost located in the same chromosome regions within the complement.

Based on certain molecular analysis, it is possible to ascribe that the basal *U. malabaricus* group is closely aligned in its affinity to primitive ichthyophid lineages. However, in order to expend linealogical connections between these two broader groups, Gower et al. (2002) and Frost et al. (2006) have proposed a possibility of an intermediate taxon.

Phylogeny of the Indian endemic genus *Uraeotyphlus* is still poorly known. But a combined approach based on morphology, biochemistry, molecular biology and cytogenetics might help to resolve a revised classification of Ichthyophiidae and thus to understand better of their phylogenetic relationships with other caecilians. Towards that effect, San Mauro et al. (2004) have attempted to provide intrafamily relationships of extant caecilians mainly based on mitochondrial genomes since they found them offering a more reliable data set for comparison. Subsequently, Zhang and Wake (2009) have extended this type of molecular analyses to include multiple gene data sets and more number of taxa. Their work on the key species *Ichthyophis malabarensis* uncovered findings favoring sisterly relationships between the genera i.e., *Ichthyophis* and *Uraeotyphlus* and thereby supporting the view that they are to a certain extent, paraphyletic in nature. The latter study have offered support to the proposition of Gower and Wilkinson (2007) that the genus *Uraeotyphlus* may be divided into the plesiomorphic *U. malabaricus* group and apomorphic *U. oxyurus*

group. The results of present study strengthen support to the derived status of *U. oxyurus* group species.

Maddin et al. (2012) have attempted to explore the possibility of utilizing morphology based upon microcomputed tomographic pictures of brain case and stape of several caecilian taxa, as an additional criterion to other morphological features that ascribed during the course of their phylogenetic assessment. However, this study seems to point towards reaching congruence thereby limiting its extent to generic level classification but not at species specificity.

While exploring probable phylogenetic relationships and rapport, it also seems possible to infer that karyological data fall in line with those of recently assimilated molecular analyses (San Mauro et al. 2004, Zhang and Wake 2009).

In conclusion, the cytogenetic study based on conventional Giemsa staining including C- and NOR bandings, upon four *oxyurus* group species of *Uraeotyphlus* taxa, indicate that they are more similar in their karyotypic profile (if not identical) which might form a monophyletic group. In the light of extensive chromosomal homology incurred, that does not preclude minor karyotypic differences necessitating in the use of other banding techniques for the improvement of karyological characterization.

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RESEARCH ARTICLE

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Cytogenetic description of the Amazonian brown brocket *Mazama nemorivaga* (Artiodactyla, Cervidae)

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Abstract
The Amazonian brown brocket *Mazama nemorivaga* (Cuvier, 1817) is a small to medium-sized deer from the Amazon rainforest and ecotones. The first karyotype described was 2n=67 to 69 + 2-7 B and FN= 69-72, in which all chromosomes were acrocentric and the X chromosome was the only submetacentric chromosome. However, important aspects of the species chromosome evolution were not resolved because of the lack of information on chromosome banding. The G-banding pattern of *M. nemorivaga* karyotype showed the presence of an XX/XY_1Y_2 sex chromosome system as a product of an X-autosome tandem fusion, which results in a basic 2n=68, FN=70 in females and 2n= 69, FN=70 in males. The fact that this karyotype only differs from that of *Capreolus capreolus pygargus* (Pallas, 1771; 2n=70, FN=72+B) by X-autosome tandem fusion may corroborate the basal condition of *M. nemorivaga* and its proximity to the ancestral karyotype of the American Odocoileini. A derived karyotype 2n=67, XY_1Y_2, FN=70 + 3B from the Brazilian state of Mato Grosso (the western Amazon) may be evidence of differentiation between western and eastern populations.

Keywords
chromosome banding, centric fusion, B chromosomes, multiple sex chromosome system

Introduction

The Amazonian brown brocket *Mazama nemorivaga* (Cuvier, 1817) is a small to medium-sized deer that possesses a large muzzle, small ears, protruding eyes, small and spike-like antlers in males and a uniform dark brown coat. This species occurs mainly in the Amazon rainforest and ecotones of Brazil, French Guiana, Surinam, Guyana, Venezuela, Colombia, Ecuador, Peru, and most likely in Bolivia. Although classified as a species of Least Concern by the Red List of the International Union for Conservation of Nature (IUCN) because of its wide distribution, the species would be seriously affected by the effects of deforestation (Rossi et al. 2010). This species belongs to the Rangiferini tribe, which was recently included within the subfamily Capreolinae (González et al. 2010). It was once classified as a subspecies of the gray brocket *M. gouazoubira superciliaris* (Cabrera, 1961), but recent molecular analyses demonstrated that *M. nemorivaga* is a clearly differentiated species, located basally in the exclusively South American clade of Odocoileinae that also groups together *Blastocerus* (Illiger, 1815), *Ozotoceros* (Linnaeus, 1758), *Hippocamelus* (Molina, 1782), and *Pudu* (Molina, 1782), and which includes *M. gouazoubira* (Fisher, 1814) (Duarte et al. 2008). Cytogenetics also support this finding, since the karyotype of *M. nemorivaga* presents as 2n=67-69 + 2-7 B and FN=69-72, with a submetacentric X, a very different karyotype from that of *M. gouazoubira*: 2n=70+0-3B and FN=70 with an acrocentric X (Rossi et al. 2010). However, the lack of studies on chromosome banding patterns in this species makes it difficult to resolve important aspects of its chromosome evolution. The present study provides much of this information through the analysis of G, C and NOR banding patterns. It also brought new insights for the understanding of the complex chromosome evolution of South American deer.

Material and methods

Seven wild-caught specimens of *M. nemorivaga* were analyzed: one male and four females from the city of Santarém (Pará State, Brazil), one male from the city of Imperatriz (Maranhão State, Brazil), which lies within the eastern Amazon, and one male from the city of Juína (Mato Grosso State, Brazil), which lies within the western Amazon (Figure 1). The males from Imperatriz and Juína and one female are currently being kept in the captive breeding facilities of the "Deer Research and Conservation Center" (NUPECCE – Núcleo de Pesquisa e Conservação de Cervídeos, at São Paulo State University's, Jaboticabal campus); the other four animals died before the analysis and the cell cultures were prepared from frozen skin samples. The sampling was not homogeneous throughout the *M. nemorivaga* distribution area due to the difficulty in capturing free-living animals and the small number of captive specimens in Brazil.

Metaphase chromosomes were obtained from lymphocyte (Moorhead et al. 1960) and fibroblasts (Verma and Babu 1995) cultures using peripheral blood and skin

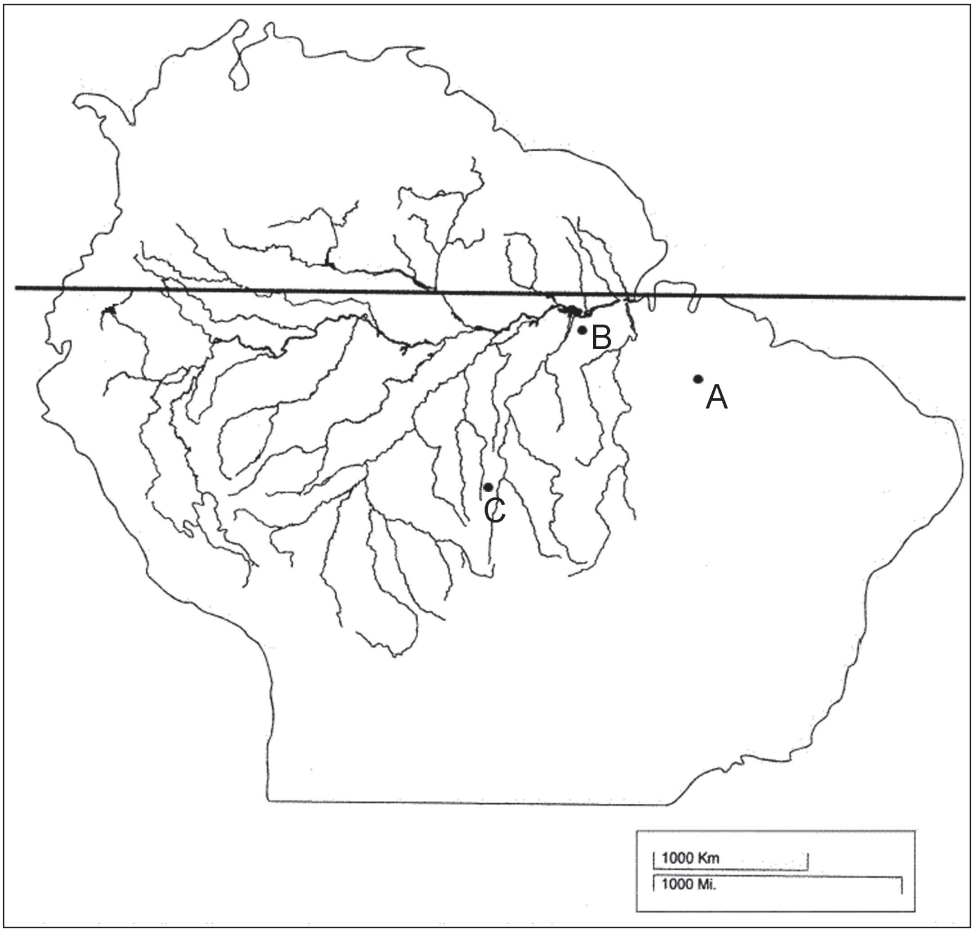

Figure 1. Origin of analyzed animals of the species *M. nemorivaga*, where A = animals from Imperatriz, MA (T265), B = animals from Santarém, PA (T261, T262, T263, T264, T266), C = animals from Juina, MT (T275, T295). Modified version of the map by Colinvaux et al. 2000.

fragments, respectively. The chromosomes were studied using G-banding (Seabright 1971), C-banding (Sumner 1972), and Ag-NOR staining (Howell and Black 1980). Approximately 40 metaphases from each specimen were analyzed in order to determine the diploid number (2n) and the fundamental number (FN). The chromosomes were classified following Abril and Duarte (2008) as metacentric, submetacentric or acrocentric according to their arm ratio, and were then organized into groups according to their relative lengths (RL): A (biarmed chromosomes with RL > 2.5%), C (biarmed chromosomes with RL < 2.5%), D (acrocentric chromosomes with RL < 3.0%), E (acrocentric chromosomes with RL > 3.0%) and B (microchromosomes or extranumerary chromosomes with RL > 1.0%). B chromosomes were not considered in the calculation of the diploid or fundamental numbers because there was intraindividual variation (Abril and Duarte 2008).

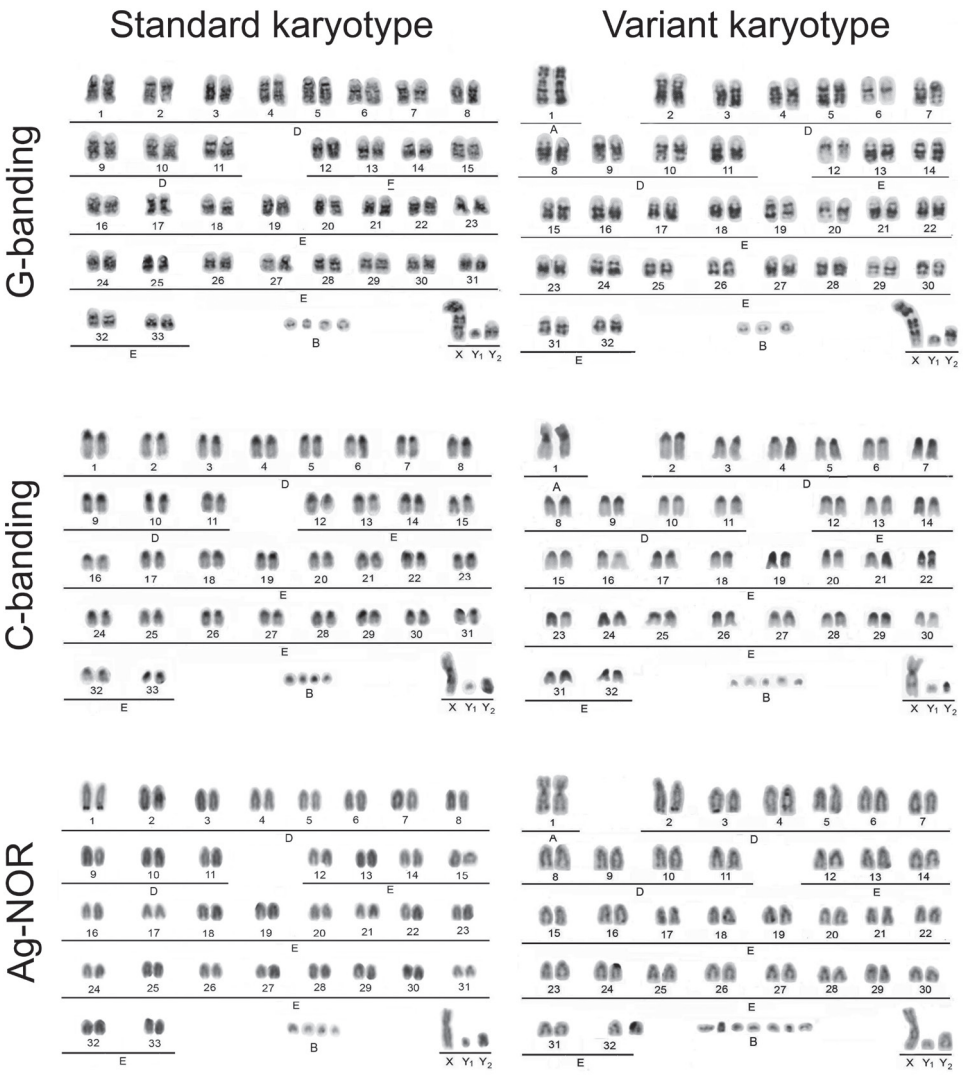

Figure 2. Standard and variant karyotypes of two *M. nemorivaga* males after G- and C-banding and Ag-NOR staining.

Results and discussion

The karyotypes of six animals from the eastern Amazon were found to be standard karyotypes with 2n=68/69 + 2-7B XX/ XY_1Y_2 FN=70, composed of 66 acrocentric autosomes from the E group, with the X chromosome being a medium-sized submetacentric chromosome and the males presenting one small metacentric Y_1 chromosome and one small acrocentric Y_2 chromosome. The male from the western Amazon had a variant karyotype with 2n=67 + 7B XY_1Y_2 FN=70, composed of one pair of large submetacentrics from the A group and 62 acrocentric autosomes from the E group; the

sex chromosomes were the same as in the males from the eastern Amazon. The large submetacentric pair in this specimen showed homology with the 4q and 32p of the standard karyotype. The X chromosome showed an interstitial C-band in the middle of the q arm, and its distal half was homologous to the Y_2 chromosome, which confirm X-autosome tandem fusion. All chromosomes except the Y_1 chromosome presented extended pericentromeric C-bands. The NOR regions were seen in autosomes as satellites that were distal to the telomeres of the q arms of the two larger acrocentrics (first and second pairs). Additionally, a number of very small supernumerary or B chromosomes, varying from two to seven chromosomes, were present in all of the animals.

The results showed a basic karyotype of 2n=68/69 + 2-6B FN=70, with a sex chromosome system of the XX/XY_1Y_2 type for *M. nemorivaga*. This kind of sex chromosome system has been widely described for several deer species of the subfamily Muntiacini (Fontana and Rubini 1990), but it is rare in the subfamily Capreoleinae; it has been reported only in the red brocket *M. americana* from Brazil (Sarria-Perea 2004, Abril et al. 2010). This rearrangement might has been achieved by both species independently, since they belong to two phylogenetically distant clades (Duarte et al. 2008, Gonzalez et al. 2010).

When comparing the karyotype of *M. nemorivaga* to the other species of the subfamily Capreoleinae, its standard karyotype seems to be more similar to that of the Eurasian roe deer species *Capreolus capreolus* (Linnaeus, 1758; with 2n=70, FN=72) and *C. c. pygargus* (with 2n=70+10B, FN=72) than to that of other phylogenetically closer South American species such as *Hippocamelus bisulcus* (Molina, 1782) (Vila et al. 2010) and *Pudu puda* (Molina, 1782), which both possess a 2n=70, FN=74 karyotype (Fontana and Rubini 1990). The karyotype of *M. nemorivaga* differs from the roe deer karyotypes only by X-autosome tandem fusion and this particular sex chromosome system can be considered as an autapomorphic feature, as the product of an independent process of chromosome evolution. This proximity to the karyotype of the roe deer agrees with molecular phylogenetics that arranges *M. nemorivaga* as a basal species onto the South American Rangiferini clade (Duarte et al. 2008, Gonzalez et al. 2010). The proximity may also suggest that the ancestral karyotype of the New World Rangiferini is likely to be 2n=70, FN=72 rather than the 2n=70, FN=70 karyotype that has previously been reported in the literature (Fontana and Rubini 1990).

The single animal from the western Amazon analyzed here had a different karyotype, which was composed of 64 autosomes, with one pair from the A group. According to its G-banding patterns, this new submetacentric pair was the product of the Robertsonian translocation between the chromosomes 4 and 32 from standard karyotype. The fixation of this kind of rearrangement in some local population increases a probability of reproductive isolation of this population. Such a process has indeed been observed in a sympatric brocket deer species *M. americana* (Abril et al. 2010).

These results seem to indicate that *M. nemorivaga* has a karyotype similar to the ancient one, but it followed an independent and complex chromosomal evolutionary pathway. The existence of polymorphic karyotypes likely indicate some degree of population differentiation and this can be found in other neotropical brocket deer species (Abril and

Duarte 2008; Abril et al. 2010). Despite the small number of analyzed animals in this study, these are the first results concerning the karyotypic status of the *M. nemorivaga* in Brazil, and they are fundamental for a new review of chromosomal evolution in Cervidae.

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RESEARCH ARTICLE

COMPARATIVE Cytogenetics
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Comparative cytogenetics of two endangered leuciscine fish, *Squalius aradensis* and *S. torgalensis* (Teleostei, Cyprinidae), from the Iberian Peninsula

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Abstract

In this study, the description of the karyotypes of the endangered chubs *Squalius aradensis* (Coelho, Bogutskaya, Rodrigues and Collares-Pereira, 1998) and *Squalius torgalensis* (Coelho, Bogutskaya, Rodrigues and Collares-Pereira, 1998) is presented by means of conventional (Giemsa-staining, Chromomycin A_3 (CMA_3)-fluorescence, Silver-impregnation (Ag-NORs)) and molecular (fluorescence *in situ* hybridization (FISH) with 18S rDNA probe) protocols. These endemic sister-species have an allopatric but adjacent distribution in the most southwestern part of the Iberian Peninsula. Diploid chromosome number was invariably 2n = 50 and karyotypes of both species were grossly similar, composed of metacentric and submetacentric elements with a reduced number of acrocentric pairs. Sequential staining using FISH with an 18S rDNA probe, CMA_3 and Ag-NORs treatments revealed consistent positive signals located at the end of the short arms of a submetacentric chromosome pair, likely homologous in both species. While providing useful cytogenetic comparative data against other members of the genus *Squalius* Bonaparte, 1837, the work aimed to draw attention towards the conservation of two narrow-range and highly confined fish species.

Keywords

Leuciscinae, Cytotaxonomy, FISH with rDNA, NOR-phenotype, silver staining, chromomycin A_3

Introduction

The genus *Squalius* Bonaparte, 1837 belongs to the subfamily Leuciscinae, the major element of the Iberian cyprinid fauna. In Portuguese inland waters, four bisexual species with an allopatric distribution (Leunda et al. 2009) are recognized: *S. pyrenaicus* (Günther, 1868), in most drainages from the Tejo River basin southwards; *S. carolitertii* (Doadrio, 1988), from the most northern smaller drainages of the Atlantic slope to the Mondego River basin; *S. aradensis* (Coelho, Bogutskaya, Rodrigues and Collares-Pereira, 1998), in the Seixe, Aljezur, Alvor, Arade and Quarteira River basins; and *S. torgalensis* (Coelho, Bogutskaya, Rodrigues and Collares-Pereira, 1998), only present in the southern Mira River basin.

Most chubs of the genus *Squalius* were formerly included in the genus *Leuciscus* Cuvier, 1816 (see Sanjur et al. 2003). The two species with a wider geographic distribution, *S. carolitertii* and *S. pyrenaicus* (also present in Spain), were the first to be recognized as different taxonomic units. However, some genetic studies (Coelho et al. 1995, Brito et al. 1997) hypothesized the existence of a more complex differentiation pattern in the most south-western Iberian populations. Two new species, with a restricted geographic range, were then described by Coelho et al. (1998), *S. aradensis* and *S. torgalensis*, mainly based on meristic, morphometric and osteological traits. Further studies using in-depth mitochondrial and nuclear markers have confirmed that *S. aradensis* and *S. torgalensis* are separated, but sister-taxa (see Doadrio and Carmona 2003, Sanjur et al. 2003, Mesquita et al. 2005, 2007, Almada and Sousa-Santos 2010, Perea et al. 2010, Waap et al. 2011).

Some cytogenetic data were recorded for populations from Arade, Aljezur and Bordeira drainages (currently assigned to *S. aradensis*) and Mira (currently assigned to *S. torgalensis*) (Collares Pereira et al. 1998), but previously to the acknowledgement that they should be considered distinct taxa from *S. pyrenaicus* (Coelho et al. 1998). Besides, Collares-Pereira et al. (1998) reported higher karyotype variability in chubs belonging to southern populations, when compared to the northern drainages. Therefore, the present work aimed to describe the karyotypes of *S. aradensis* and *S. torgalensis* using specimens recently collected. Conventional (Giemsa staining, GC-specific CMA_3 fluorescence and Ag impregnation) and molecular (rDNA major complex by FISH) cytogenetic techniques were used to characterize both species chromosome sets. Both endemics are "Critically Endangered" (Cabral et al. 2005) and besides an inherent heuristic nature, this work might draw attention towards the conservation of two narrow-range and highly confined fish species, while withdrawing useful cytogenetic comparative data against other members of the genus *Squalius*.

Material and methods

Adult specimens were captured by electrofishing in two distinct southern populations. Six *S. aradensis* (four males and two females) were collected in the Arade basin (Ode-

louca River) and two *S. torgalensis* (unknown sex) in the Mira basin (Torgal River). All procedures were developed in accordance to the recommended ethic guidelines (ASAB 2006). Some old chromosome images from material collected in the same drainages and studied by Collares-Pereira et al. (1998) were also reanalyzed (2 specimens of *S. aradensis* and 3 specimens of *S. torgalensis*) in order to confirm the results obtained with the new material.

Chromosome spreads were obtained for one specimen using standard kidney protocol and for the remaining using fin fibroblast cultures (Rodrigues and Collares-Pereira 1996) to avoid fish sacrifice. Chromosome spreads were obtained by conventional splashing and selected for further cytogenetic analysis.

Chromosomes were stained with a solution of 4% Giemsa (pH=6.8). CMA_3 fluorescence staining was performed according to Sola et al. (1992), with a slide pre-wash in McIlvaine/$MgCl_2$ buffer, one hour incubation with CMA_3 (Calbiochem) and Methyl green counterstaining. Ag-NORs detection followed Howell and Black (1980) with modifications (Gold and Ellison 1983), using Giemsa counterstaining. Whenever possible, slides were destained and used in sequential treatments.

The physical mapping of major rDNA gene cluster on the chromosomes was accomplished by FISH with rDNA probe. An rDNA clone containing 18S-5.8S-28S genes plus the intergenic spacers and untranscribed sequences from the genome of *Drosophila melanogaster* Meigen, 1830 (clone pDm 238, Rohia et al. 1981) was used as probe and labelled by nick translation with digoxigenin-11-dUTP according to the manufacturer's specifications (Roche Applied Science). The probe was resuspended in hybridization mix composed of 50% ultra-pure formamide pH=7.5 (Sigma-Aldrich), 2× SSC and 10% (w/v) dextran sulfate powder (Sigma-Aldrich). Slides were denaturated in 70% formamide in 2× SSC at 65°C for 3 minutes, and immediately dehydrated in an ice-cold ethanol series (70%-90%-100%) for 7 minutes each and air-dried. The probe mixture was denatured at 75°C for 10 minutes, immediately placed on ice for another 10 minutes and added to the chromosome preparation. Hybridization was performed overnight in a dark moist chamber at 37°C. Post-hybridization washes were performed at room temperature (RT), for 7 minutes each: twice in 2× SSC and once in 2× SSC/0.1% $Tween_{20}$. Slides were incubated with 3% bovine serum albumin (BSA) for 30 minutes at 37°C, in a dark moist chamber. Anti-digoxigenin antibody conjugated with fluorescein isothicyanate (FITC) (Roche Applied Science) was used to detect the probe for an hour and a half incubation at 37°C in a moist chamber. The slides were washed twice in 1x Phosphate Buffered Saline solution (PBS) at RT for 7 minutes and counterstained with DAPI in antifade solution.

Slides were screened in an Olympus BX 60 epifluorescence microscope equipped with a DP50 Olympus CCD camera. All images were processed using Adobe Photoshop CS4 software. Chromosomes were arranged in a decreasing size order and classified according to their arm ratios (Levan et al. 1964) in three categories: metacentric (m), submetacentric (sm) and sub-telocentric to acrocentric (st/a). To determine the fundamental number (NF value), chromosomes of the m and sm groups were considered biarmed and those of group st/a as uniarmed.

Results

All *S. aradensis* and *S. torgalensis* karyotypes revealed a diploid number of 2n = 50 chromosomes.

Karyotypes of both *S. aradensis* and *S. torgalensis* are composed of five pairs of metacentric (m), eighteen pairs of submetacentric (sm) and two pairs of subtelo/acrocentric (st/a) chromosomes. As the general karyotypes are the same for both species, only the results for *S. aradensis* using giemsa staining were included in Fig. 1. This genome composition leads to a high fundamental number (NF=96). As regards the eventual occurrence of heteromorphic sex chromosomes, no clear distinction between male and female karyotypes was observed in *S. aradensis*, the species where the specimens' sex could be accurately assessed.

The NORs' phenotype was constant throughout all treatments, consistently positively labeling only one NORs-bearing chromosome pair likely homologous in both *S. aradensis* and *S. torgalensis* species. Their rDNA-positive signals were co-localized to CMA_3- and Ag-positive signals: in the short arm of a middle-size sm chromosome pair in both species as documented by sequential staining (Figs 2 and 3), indicative of being GC-rich and transcriptional active. No evidences of multi-chromosomal positive NOR's signals were registered.

Discussion

S. aradensis and *S. torgalensis* karyotypes display the general pattern described for most Leuciscinae: a diploid chromosome number of 2n = 50, a chromosome set with mainly bi-armed elements (dominated by submetacentrics with some metacentrics), and only one NOR-bearing chromosome pair. In cyprinids this is assumed to correspond to the ancestral character state and most likely represents also the synapomorphy for the genus *Squalius* (Ráb and Collares-Pereira 1995). A large subtelocentric/acrocentric

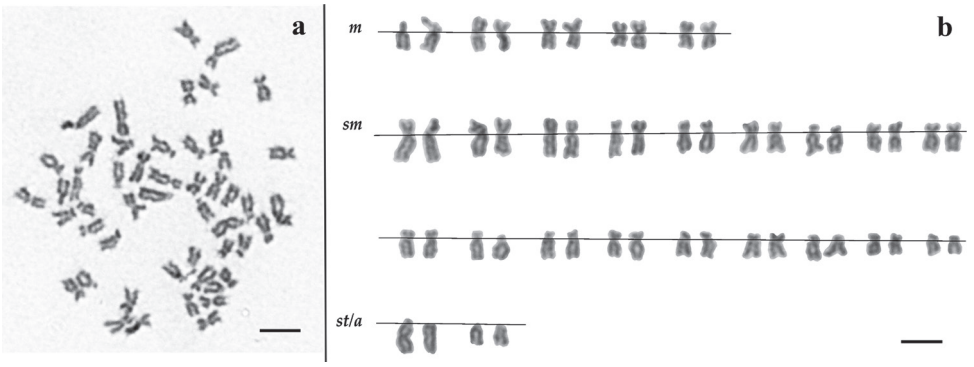

Figure 1. a Giemsa stained metaphase and **b** corresponding karyotype of a *S. aradensis* male from Odelouca River (Arade drainage). Scale bar = 10μm.

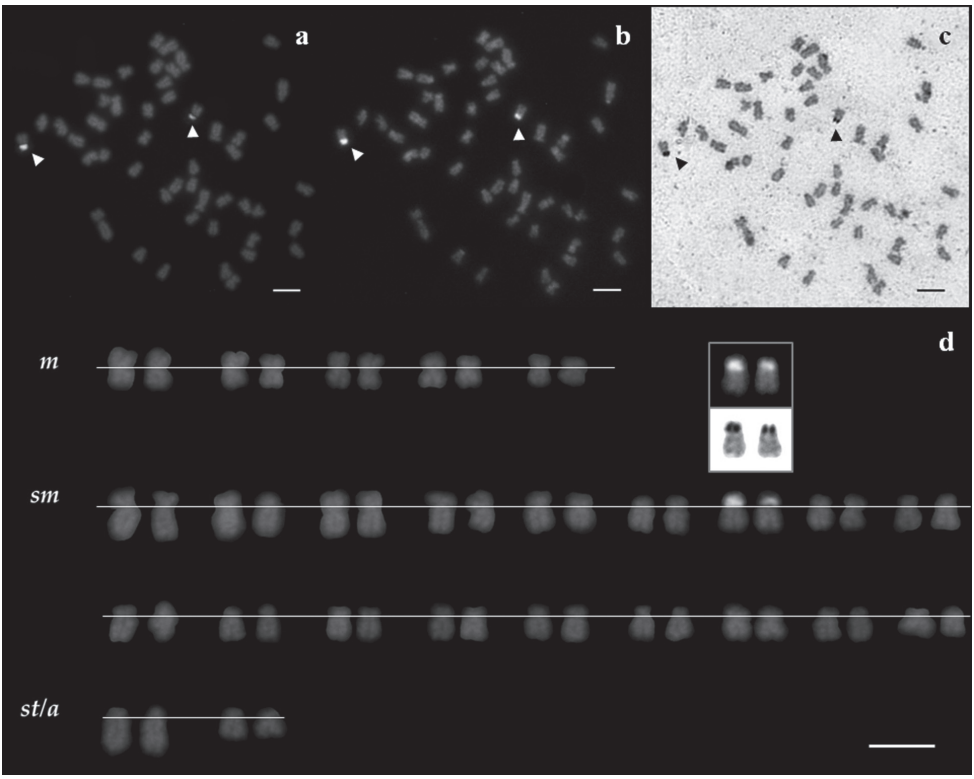

Figure 2. Metaphase spreads of a *S. aradensis* male, stained with sequential treatments **a** rDNA FISH **b** CMA_3-NOR, and **c** Ag-NOR treatments **d** corresponding karyotype of the same individual after rDNA FISH, with CMA_3- and Ag-NOR signals inset. NORs are indicated by arrowheads. Scale bar = 10μm.

pair previously considered a chromosome marker for the subfamily (Ráb and Collares Pereira 1995, Ráb et al. 2008) is also present in both species. The most common situation found in European Leuciscinae of a slightly higher number of st/a chromosomes might be due to the difficulty in obtaining high quality images for an accurate classification of chromosomes (Ráb and Collares-Pereira 1995, Collares-Pereira et al. 1998, Boron et al. 2009).

The number and location of rDNA gene clusters have been used as chromosome markers in fish cytotaxonomy (e.g. Rábová et al. 2003, Boron et al. 2009, Kirtiklis et al. 2010, Pereira et al. 2012, Rossi et al. 2012). Conversely to what was reported in *S. pyrenaicus* (Gromicho et al. 2005), no NORs polymorphism was observed in this study. Besides, Collares-Pereira et al. (1998) did not report any variation in NORs' phenotype in all the populations analyzed. Karyotypes of these species conserved the plesiomorphic condition, where leuciscines have, in general, only one NOR-bearing chromosome pair (Ráb and Collares-Pereira 1995, Bianco et al. 2004, Luca et al. 2010, Rossi et al. 2012). Multiple NORs sites were detected in some *S. pyrenaicus* individuals using 28S rDNA FISH mapping, presenting intra-individual variation and also failure in Ag-NOR in detecting most rDNA copies (Gromicho et al. 2005). However, those

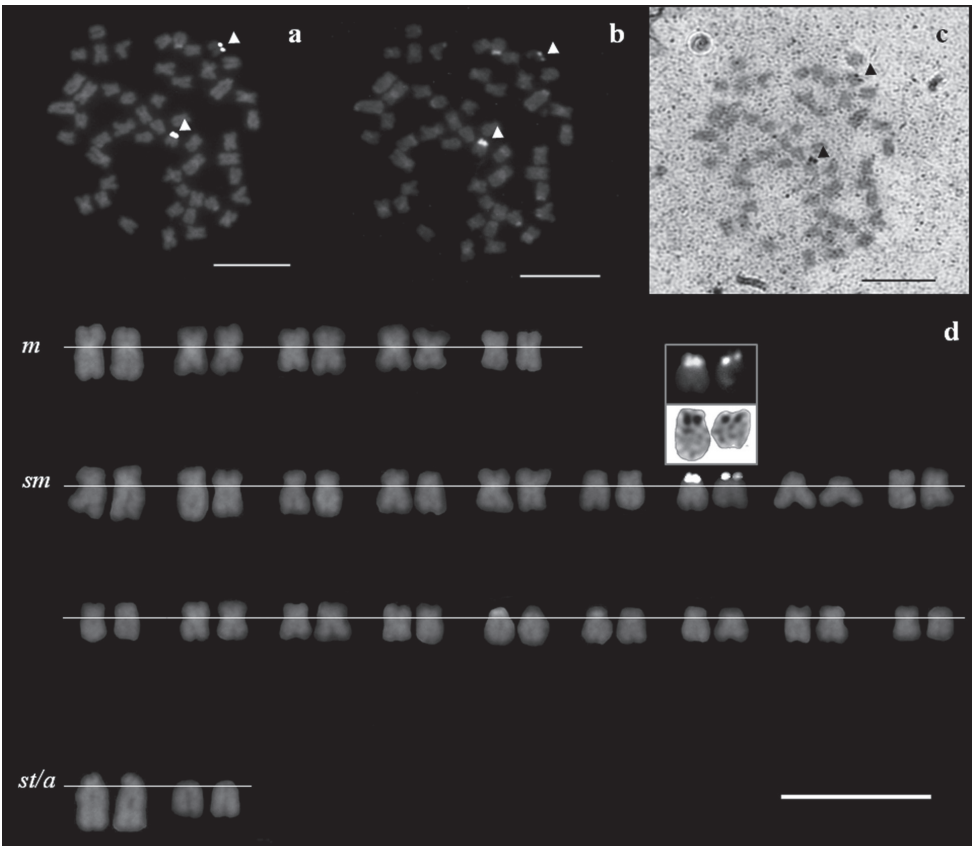

Figure 3. Metaphase spreads of a *S. torgalensis*, stained with sequential treatments **a** rDNA FISH **b** CMA_3-NOR, and **c** Ag-NOR treatments **d** corresponding karyotype of the same individual after rDNA FISH, with CMA_3- and Ag-NOR signals inset. NORs are indicated by arrowheads. Scale bar = 10μm.

observations were made in specimens living in sympatry with the *S. alburnoides* hybridogenetic complex (reviewed in Collares-Pereira and Coelho 2010) where hybridization is a recurrent process and might thus potentiate such variation in NORs.

The karyotypes of the two species proved to be grossly similar at a macrostructural level. They are sister-taxa strongly clustered in many phylogenetic analyses, however consistently reciprocally monophyletic (Almada and Sousa-Santos 2010, Waap et al. 2011). Given their differentiation estimated at 7-8 MY ago (Doadrio and Carmona 2003, Sousa-Santos et al. 2007, Almada and Sousa-Santos 2010), perhaps more subtle chromosome differences will be found when new cytogenetic tools with a higher resolution will be operating in cyprinid's chromosomes. The clear segregation of their lineage to the one including *S. pyrenaicus* and *S. carolitertii* is also consensual in phylogenetic analyses, and tree topology strongly supports them as basal members of the south-western Iberian *Squalius* lineage. Taking that into consideration, it is not surprising the conservation of the plesiomorphic state of the karyotype in both *S. aradensis*

and *S. torgalensis* but their differentiation to the other two species living in Portuguese inland waters, respectively with 10–12m + 30–32sm + 8st/a for *S. carolitertii*, and 12m + 32sm + 6st/a for *S. pyrenaicus* (Collares-Pereira et al. 1998).

Despite the apparent conservative pattern found in the two species here addressed by conventional cytogenetic tools, the karyotype variability present in the Iberian species of the genus *Squalius* so far analysed (Collares-Pereira et al. 1998, Gromicho et al. 2005), supports the occurrence of speciation processes favored by drastic changes in hydrological regimes (namely drought events), hence the difference between northern and southern populations. Whenever isolation and population bottlenecks occur, the sporadic mass reductions of population size might contribute to the stochastic fixation of chromosomal and genome mutations (Pereira et al. 2012). In particular, *S. torgalensis* is geographically confined to a single intermittent river system and characterized by a very low level of genetic diversity (see also Almada and Sousa-Santos 2010, Henriques et al. 2010). Thus specific conservation measures have to be adopted if the option will be to preserve the genome integrity of this highly vulnerable species.

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RESEARCH ARTICLE

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Comparative cytogenetics of two species of genus *Scobinancistrus* (Siluriformes, Loricariidae, Ancistrini) from the Xingu River, Brazil

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Abstract

The family Loricariidae encompasses approximately 800 species distributed in six subfamilies. The subfamily Hypostominae consists of five tribes; of them, the tribe Ancistrini is relatively diverse, but it is not well known from the cytogenetic point of view. Genus *Scobinancistrus* Isbrücker et Nijssen, 1989, which is part of the tribe Ancistrini, has two species that occur in sympatry in the Xingu River, Brazil. In this work, we performed the first karyotypic characterizations of these two species and sought to identify the processes involved in their karyotypic evolution. Chromosomal preparations were subjected to Giemsa staining, silver nitrate impregnation, C-banding, CMA_3 staining, DAPI staining, and FISH (fluorescence *in situ* hybridization) with 18S rDNA and telomeric probes. We found that *S. aureatus* Burgess, 1994 and *S. pariolispos* Isbrücker et Nijssen, 1989 shared the diploid number, 2n=52, but differed in their karyotypic formulae (KFs), distribution of constitutive heterochromatin (CH), and the localizations of their nucleolus organizer regions (NORs), which were found on the interstitial and distal regions of the long arm of chromosome pair 3 in *S. aureatus* and *S. pariolispos* respectively. We suggest that these interspecific variations may have arisen via paracentric inversion or transposition of the NOR. The karyotypic differences found between these two *Scobinancistrus* species can be used to identify them taxonomically, and may have functioned as a mechanism of post-zygotic reproductive isolation during the speciation process.

Keywords

Karyotypic divergence, chromosome rearrangements, sympatry

Introduction

The fishes of the family Loricariidae are an important component of the ichthyofauna in the Neotropical region, where they are widely distributed and occupy a great variety of freshwater environments (Isbrücker 1980). The 800 known species are organized into six subfamilies: Hypoptopomatinae, Hypostominae, Lithogeninae, Loricariinae, Neoplecostominae and Delturinae (Armbruster 2004, Reis et al. 2006). The subfamily Hypostominae encompasses five tribes, Corymbophanini, Rhinelepini, Hypostomini, Ancistrini and Pterygoplichthyini, with the latter two forming the most derived clade (Armbruster 2004). The Ancistrini include numerous species with several yet-unsolved taxonomic problems, making it difficult to recognise them (Alves et al. 2003).

Cytogenetic information is incipient, given the great diversity of Ancistrini species, and has been efficient in distinguishing species of this tribe (Alves et al. 2003, Souza et al. 2009). It is therefore necessary to increase the amount of such information in order to improve the taxonomic identification of these fishes and understanding the evolutionary processes in this group. The members of the tribe Ancistrini have diploid numbers that vary from 2n=34 in *Ancistrus* sp. from the Purus river to 2n=52 in most of the other species karyotyped to date. Several sex chromosome systems have been identified, including simple and multiple systems, with either the male or the female as heterogametic sex. The numbers and localizations of the nucleolus organizer regions (NORs) are also rather variable; some species have simple NORs while others have multiple NORs (Artoni and Bertollo 2001, Alves et al. 2003, 2006, Souza et al. 2004, 2009, De Oliveira et al. 2006, 2007, 2008, 2009).

The genus *Scobinancistrus* Isbrücker et Nijssen, 1989 (Hypostominae, Ancistrini) comprises two species: *S. pariolispos* Isbrücker et Nijssen, 1989, which occurs in the Tapajós, Xingu and Tocantins Rivers, Brazil, and *S. aureatus* Burgess, 1994, which is endemic to the Xingu River (Fisch-Muller 2003, Camargo et al. 2004). They differ in that *S. pariolispos* has a fully evertible operculum, a final ray of the dorsal fin that extends as far as the adipose, and fins with no orange coloration (Camargo et al. 2012). No previous study has provided cytogenetic information for the two species of this genus.

In the present work, we obtained *S. pariolispos* and *S. aureatus* from the Xingu River and studied their karyotypes, in an effort to identify the processes involved in their karyotypic evolution and contribute new cytogenetic information for members of the tribe Ancistrini.

Material and methods

Samples of *Scobinancistrus aureatus* (seven females) and *S. pariolispos* (five males and two females) from the Xingu River, Brazil, were analyzed (Fig. 1). Metaphase chromosomes were obtained according to the method described by Bertollo et al. (1978) and analyzed by conventional staining (Giemsa), C-banding (Sumner 1972), Ag-NOR labeling (Howell and Black 1980), chromomycin A_3 staining (Schweizer 1980), DAPI

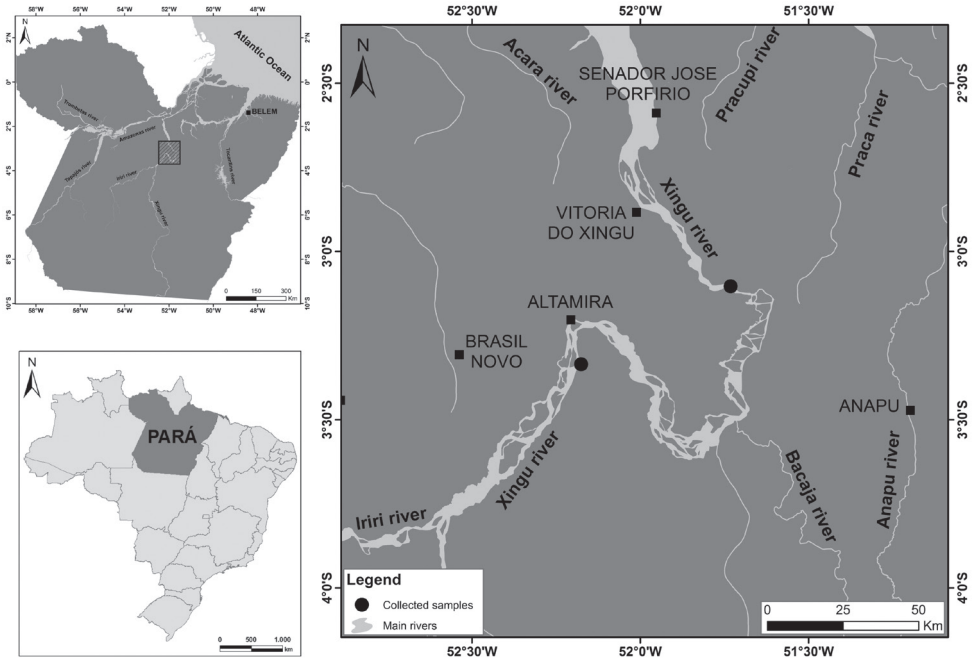

Figure 1. Collection localities of the analyzed *Scobinancistrus* samples.

staining (Pieczarka et al. 2006), and fluorescence *in situ* hybridization (FISH) with 18S ribosomal DNA (rDNA 18S) probes obtained from *Prochilodus argenteus* Agassiz, 1829 (Hatanaka and Galetti 2004) and human telomeric sequence probes (Oncor). The probes were labeled with biotin or digoxigenin by nick translation and detected with avidin-CY3 or anti-digoxigenin-FITC. The chromosomes were arranged according to the procedure described by Levan et al. (1964).

Results

The specimens of *S. aureatus* and *S. pariolispos* obtained from the Xingu River both had diploid numbers 2n=52 chromosomes, but they differed in their karyotypic formulas (KFs), which were 22m-20sm-10st and 24m-18sm-10st, respectively (Fig. 2A, B). Both males and females were analyzed for *S. pariolispos*, but no sex chromosomes were identified.

C-banding failed to identify constitutive heterochromatin (CH) in the centromeric region of any chromosome in the studied species. In *S. aureatus*, large heterochromatic blocks were seen, as follows: in the proximal regions of the long arms of chromosome pairs 5, 6 and 18; in the proximal regions of the short arms of pair 13; throughout the short arms of pair 22; in the distal regions of the long arms of pairs 3 and 12; and in the distal regions of the short arms of pairs 12 and 18 (Fig. 2C). In *S. pariolispos*, conspicuous heterochromatic blocks were also identified throughout the

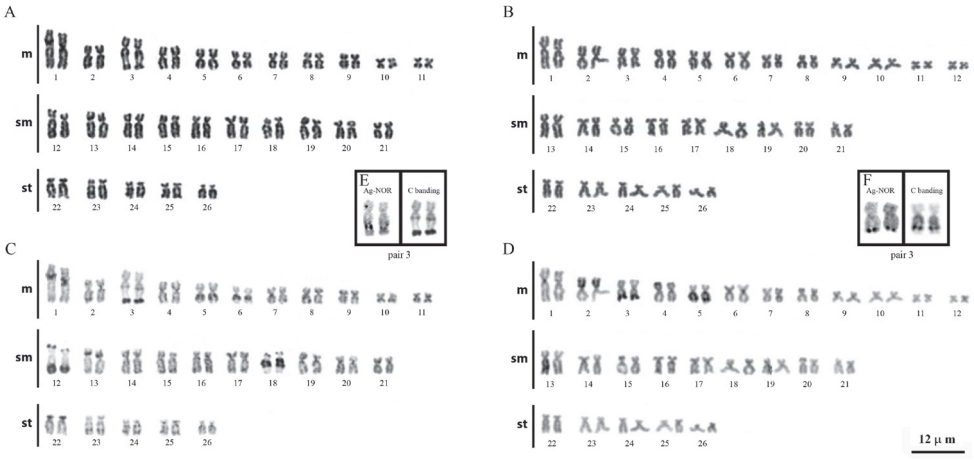

Figure 2. Karyotypic analyses of *S.aureatus* (**A, B** and **E**) and *S. pariolispos* (**B, D** and **F**): conventional staining (**A** and **B**), C-banding (**C** and **D**) and the NOR-bearing chromosome pair (**E** and **F**). The scale bar refers to all images.

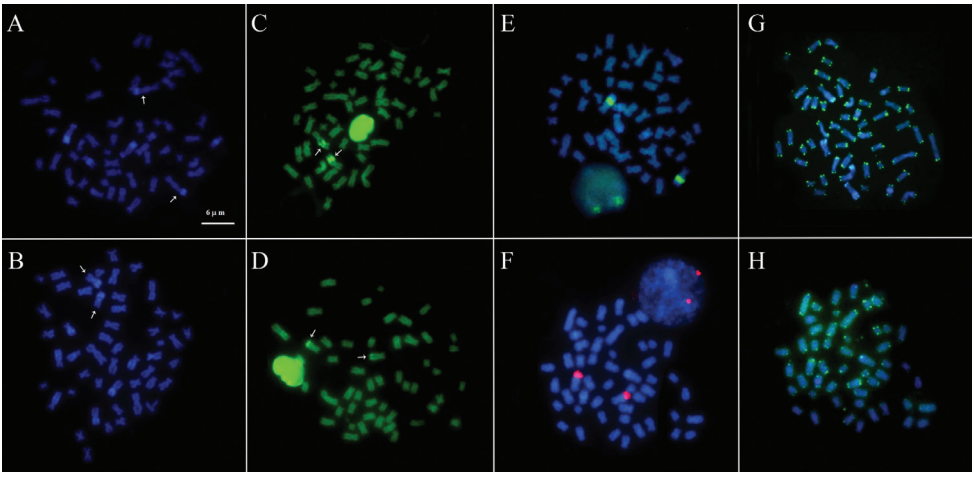

Figure 3. Further karyotypic analyses of *S. aureatus* (**A, C, E** and **G**) and *S. pariolispos* (**B, D, F** and **H**): DAPI staining (**A** and **B**), CMA_3 staining (**C** and **D**), FISH with 18S rDNA probes (**E** and **F**) and FISH with telomeric sequence probes (**G** and **H**). The scale bar refers to all images.

short arms of pair 2, in the distal regions of the long arms of pair 3, and throughout the long arms of pair 5 (Fig. 2D).

A NOR was identified on a single chromosome pair per species. In *S. aureatus*, the NOR was located in the interstitial region of the long arms of pair 3, flanked by CH (Fig. 2E). In *S. pariolispos*, the NOR was situated in the distal region of the long arms of pair 3, adjacent to a block of CH (Fig. 2F). The numbers and localizations of these regions were confirmed by FISH with 18S ribosomal DNA probes (Fig. 3E, F).

The DAPI fluorochrome labeled the heterochromatic regions (Fig. 3A, B), and CMA_3 stained the NORs (Fig. 3C, D). In FISH using telomeric sequence probes (TTAGGG), the probes hybridized to the ends of all chromosomes, but no interstitial telomeric labeling was observed (Fig. 3G, H).

Discussion

Scobinancitrus aureatus and *S. pariolispos* from the Xingu River were found to have the same diploid number (2n=52), but their KFs differed. This is consistent with most other species of tribe Ancistrini, which share 2n=52 and differ in their KFs (Artoni and Bertollo 2001, Alves et al. 2003, 2006, Souza et al. 2004, 2009, De Oliveira et al. 2007). Chromosomal inversions, which are rearrangements that can modify the structure of chromosomes without altering their number, may explain how these species have the same diploid number but different KFs (Alves et al. 2003, De Oliveira et al. 2006). According to Artoni and Bertollo (2001) and Kavalco et al. (2005), 2n=54 probably corresponds to a basal condition for the Loricariidae. Therefore, the reduction to 2n=52 in most of the Ancistrini species must be the result of a fusion event. The same type of rearrangement is believed to explain the reduction of the diploid number in several species of the genus *Ancistrus* Kner, 1854 (Alves et al. 2003, De Oliveira et al. 2009).

The NOR was found on the same chromosome pair in the two *Scobinancistrus* species studied here in this situation is shared by most members of the tribe Ancistrini (Artoni and Bertollo 2001, Alves et al. 2003, 2006, De Oliveira et al. 2007, Souza et al. 2004, 2009). The long arm of chromosome pair 3 was identified as the bearer of the NOR in both species; however, it was found in an interstitial, CH-flanked region in *S. aureatus*, but in the distal region, adjacent to a CH block, in *S. pariolispos*. This organization indicates that the NOR-bearing chromosomes are not the same in *S. aureatus* and *S. pariolispos*, suggesting the occurrence of events that changed the position of the NOR within the karyotype (Fig. 4). The repetitive nature of the ribosomal DNA that constitutes the NOR and its association with CH (which consists mainly of satellite DNA and transposable elements; Dimitri et al. 2009) may facilitate transposition events that can move the NOR to another region of the genome (Gross et al. 2009, 2010). It is important to point out that the determination of chromosome pair numbers in these species is tentative, and therefore pair 3 of one species is not necessarily homologous to pair 3 of another species. However, we cannot exclude the possibility that these pairs are homologous and reflect the occurrence of a paracentric inversion involving the NOR and nearby HC. The NORs labeled positive for the fluorochrome, CMA_3, but negative for DAPI, indicating that the ribosomal DNA is interspersed with repetitive GC-rich DNA, as has been frequently described in other fishes (Pendás et al. 1993).

The constitutive heterochromatin stained positive with DAPI, indicating that it is AT-rich. Its patterns differed between the two species, with large heterochromatic blocks in non-centromeric regions located predominantly in non-homologous chromo-

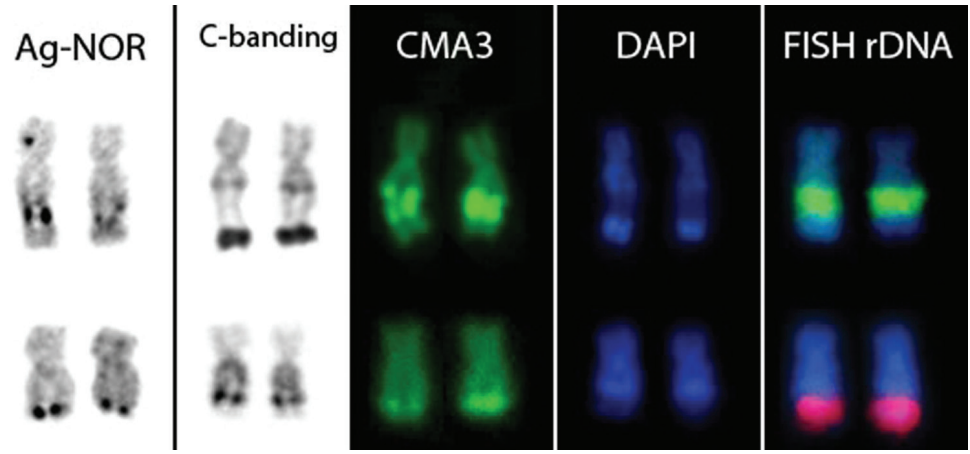

Figure 4. NOR-bearing chromosomes of *S. aureatus* (upper row) and *S. pariolispos* (lower row).

somes, indicating that processes related to the dynamics of repetitive DNA (e.g., transposition) may have been involved in the karyotypic differentiation of these two species.

Using information on karyotype macrostructures and CH and NOR distribution, Souza et al. (2009) identified chromosomes with possible homologies among species of genus *Peckoltia* Miranda Ribeiro, 1912, which belong to the tribe Ancistrini. However, using same criteria, we were unable to identify any homologies between the karyotypes of *S. aureatus* and *S. pariolispos*. This suggests that the studied karyotypes may have undergone both inversions (as noted above) and reciprocal translocation events, leading to greater genomic reorganization. Consistent with this, Nagamachi et al. (2010) used chromosome painting to demonstrate that the differences between two *Gymnotus carapo* Linnaeus, 1758 populations involved a greater number of chromosome rearrangements than previously assumed based on classical cytogenetic data (Milhomem et al. 2008).

In conclusion, the karyotypic differences found in the two *Scobinancistrus* species studied herein can be used in their taxonomic identification. Moreover, the sympatric occurrence of these species suggests that the identified karyotypic differences may have functioned as a mechanism of post-zygotic reproductive isolation during the speciation process.

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Cytogenetic studies in four cultivated *Amaranthus* (Amaranthaceae) species

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Abstract

In the present study, the chromosomes numbers were confirmed, 2n = 34 for *Amaranthus cruentus* Linnaeus, 1759, and 2n = 32 for *A. hypochondriacus* Linnaeus, 1753, *A. mantegazzianus* Passer, 1864, and *A. caudatus* Linnaeus, 1753. The distribution and variability of constitutive heterochromatin were detailed using DAPI-CMA_3 banding technique. The position of the nucleolus organizer region (NOR) was observed using Ag-NOR banding (active loci) and fluorescent *in situ* hybridization (rDNA-FISH) in the four *Amaranthus* species. Variations in the amount of constitutive heterochromatin were detected both within the species and between them, with DAPI-CMA_3 stain. One chromosome pair having a NOR was found in each studied accession, with exception of *A. caudatus* cv. EEA INTA Anguil. This accession presented four rDNA loci (FISH), being active two of them (Ag- banding).

Keywords

Amaranthus, constitutive heterochromatin, NORs, FISH, DAPI-CMA_3

Introduction

The genus *Amaranthus* Linnaeus,1753 comprises about 50 herbaceous species, most of them are annuals. They grow preferentially in warm regions of America. Several species are cultivated as ornamentals (*A. caudatus* Linnaeus,1753), vegetables (*A. spinosus* Linnaeus, 1753 and *A. tricolor* Linnaeus, 1753), pseudocereals (*A. cruentus* Linnaeus, 1759*, A. hypochondriacus* Linnaeus, 1753*, A. mantegazzianus* Passer, 1864*,* and *A. caudatus* Linnaeus, 1753), and some of them are weeds.

Karyotypical studies in the genus are scarce, probably due to the small size of the chromosomes, which makes morphological analysis difficult (Grant 1959 a, b, c). Updated data have indicated that there are two basic chromosome numbers, x = 16 and x = 17, and, in some cases, both numbers were cited for the same species (Grant 1959 a, Pal and Khoshoo 1972, Pal 1972,1973 a, b, Pal et al. 1982, Poggio 1988). Pal et al. (1982) suggested that the gametic number n = 17 originates from n = 16 through primary trisomy. Greizerstein and Poggio (1992) supported this hypothesis through the analysis of meiotic behavior of species and interspecific hybrids.

Studies carried out on chromosome morphology of some species of the genus have indicated variation in number of chromosome pairs with satellites. Palomino and Rubí (1991) reported the karyotypic formula in some cultivars of *A. hypochondriacus* and *A. cruentus,* suggesting the existence of six to ten pairs of chromosomes with satellites in different cultivars. Greizerstein and Poggio (1994) proposed karyotypic formulae of various accessions of cultivated species (*A. cruentus*, *A. hypochondriacus*, *A. mantegazzianus* and *A. caudatus*). In all studied species, only one pair of chromosomes with a satellite was found (Greizerstein and Poggio 1994). Kolano et al. (2001), indicated for two cultivars of *A. caudatus* the presence of one and two pairs of chromosomes with ribosomal hybridization signals using FISH technique with 45s ribosomal probes.

In the present work, the distribution and variability of constitutive heterochromatin and the number of active ribosomal organizer regions were studied in two different cultivars of the species *A. cruentus* (2n = 34), *A. mantegazzianus* (2n = 32), *A. hypochondriacus* (2n = 32), and *A. caudatus* (2n = 32). The aim of the study was to increase the knowledge about the genetic variability of the *Amaranthus* genus.

Material and Methods

Analyses were performed in eight accessions of *Amaranthus*. Species cultivars and origin of the studied material are listed in Table 1.

Root tips from germinated seeds were pre-treated with colchicine at room temperature for 2 h, fixed in ethanol/glacial acetic acid 3:1 (v/v) for 24 hours and stored in ethanol 70% at -20 °C. Root tips were digested using a solution containing 2% cellulase and 20% pectinase (both w/v) for 90 min at 37 °C and dissected in 45% (v/v) aqueous acetic acid, squashed under a coverslip subsequently removed by freezing in dry ice, air dried and then stored in -20°C until use.

Table 1. Species of *Amaranthus* analyzed with their respective cultivar.

Species	Cultivar	Grant by:
Amaranthus cruentus L.	Don Guiem, La Pampa.	Ing. Agr. Rosa M. de Troiani
Amaranthus cruentus L.	INDEAR SA	Dr. Francisco Trucco
Amaranthus hypochondriacus L.	Artaza	Ing. Agr. Rosa M. de Troiani
Amaranthus hypochondriacus L.	INDEAR SA	Dr. Francisco Trucco
Amaranthus mantegazzianus Passer.	Don Manuel	Ing. Agr. Rosa M. de Troiani
Amaranthus mantegazzianus Passer.	INDEAR SA	Dr. Francisco Trucco
Amaranthus caudatus L.	EEA INTA Anguil, La Pampa.	Ing. Agr. Guillermo Covas
Amaranthus caudatus L.	INDEAR SA	Dr. Francisco Trucco

Fluorochrome banding DAPI-CMA_3: Fluorochrome banding was performed in all cultivars according to Deumling and Greilhuber (1982). Slides were double stained with DAPI (4'-6-diamidino-2-phenylindole) and CMA_3 (Chromomycin A_3) and mounted in 1:1 (v/v) McIlvaine´s pH7 buffer-glycerol.

FISH: FISH was performed on mitotic cells according to Cuadrado and Jouve (1994) with minor modifications. The probe that was used is the pTa 71, that contains 9 kilobase (kb) *Eco*R1 repeat unit of 18S-5.8S-25S rDNA loci and spacers isolated from wheat, *Triticum aestivum* (Gerlach and Bedbrook 1979). Probe was labelled by nick translation with biotin 14- dUTP (Bionick Labelling System).

Slides were counterstained with 4', 6-diamidino-2- phenylindole (DAPI) (1 μg McIlvaine's citrate buffer/mL, pH = 7) for 10 min at room temperature, and subsequently mounted in antifade solution and examined with a Leica epifluorescence microscope with appropriate filters. Photographs were taken using a digital camera.

Ag-staining: Silver staining technique was carried out according with Neves et al. (1997).

Results

Interstitial bands CMA_3+/DAPI+ showed differences among cultivars (Fig. 1 and Table 2). The species *A. cruentus* cv. Don Guiem and cv. INDEAR showed two DAPI-/CMA_3+ bands. Moreover, *A. cruentus* cv. INDEAR presented eight DAPI+/CMA_3+ bands (Figs 1a and b).

The species *A. hypochondriacus* cv. Artaza showed two DAPI+/ CMA_3+ bands, while the cv. INDEAR had six DAPI+/ CMA_3+ bands (Figs 1c and d).

In *A. mantegazzianus* species cv. INDEAR showed two bands of DAPI-/CMA_3+ and two DAPI+/CMA_3+ bands, while in cv. Don Manuel only two DAPI-/CMA_3+ bands were revealed (Figs 1e and f).

Amaranthus caudatus cv. EEA INTA Anguil and cv. INDEAR had four DAPI-/CMA_3+ bands and two DAPI+/CMA_3+ bands (Figs 1g and h).

FISH (Fig. 2) revealed two ribosomal hybridization signals for all studied cultivars except *A. caudatus* cv. EEA INTA Anguil (Fig. 2d) which presented four of them.

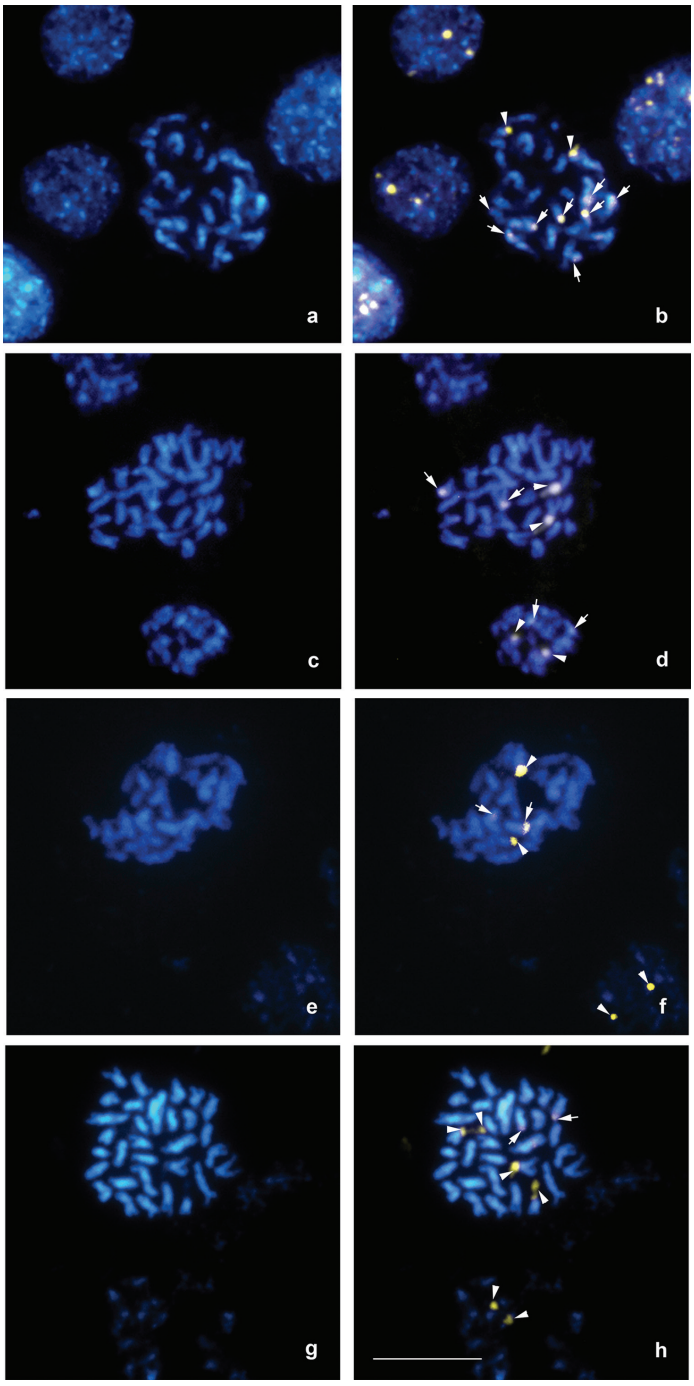

Figure 1. Metaphase cells with CMA_3/DAPI banding **a** DAPI **b** CMA_3 of *Amaranthus cruentus* cv. INDEAR **c** DAPI **d** CMA_3 of *A. hypochondriacus* cv. Artaza **e** DAPI **f** CMA_3 of *A. mantegazzianus* cv. INDEAR **g** DAPI and **h** CMA_3) of *A. caudatus* cv. INDEAR. The arrow indicates CMA_3+ band, and the arrowhead CMA_3+/DAPI+ band. Bar = 5 μm.

Table 2. Variation in DAPI/CMA_3 banding.

Species	Cultivate	Band DAPI-/CMA_3+	Band DAPI+/CMA_3+
Amaranthus cruentus	Don Guiem	2	-
Amaranthus cruentus	INDEAR SA	2	8
Amaranthus hypochondriacus	Artaza	2	2
Amaranthus hypochondriacus	INDEAR SA	2	6
Amaranthus mantegazzianus	Don Manuel	2	-
Amaranthus mantegazzianus	INDEAR SA	2	2
Amaranthus caudatus	EEA INTA Anguil	4	2
Amaranthus caudatus	INDEAR SA	4	2

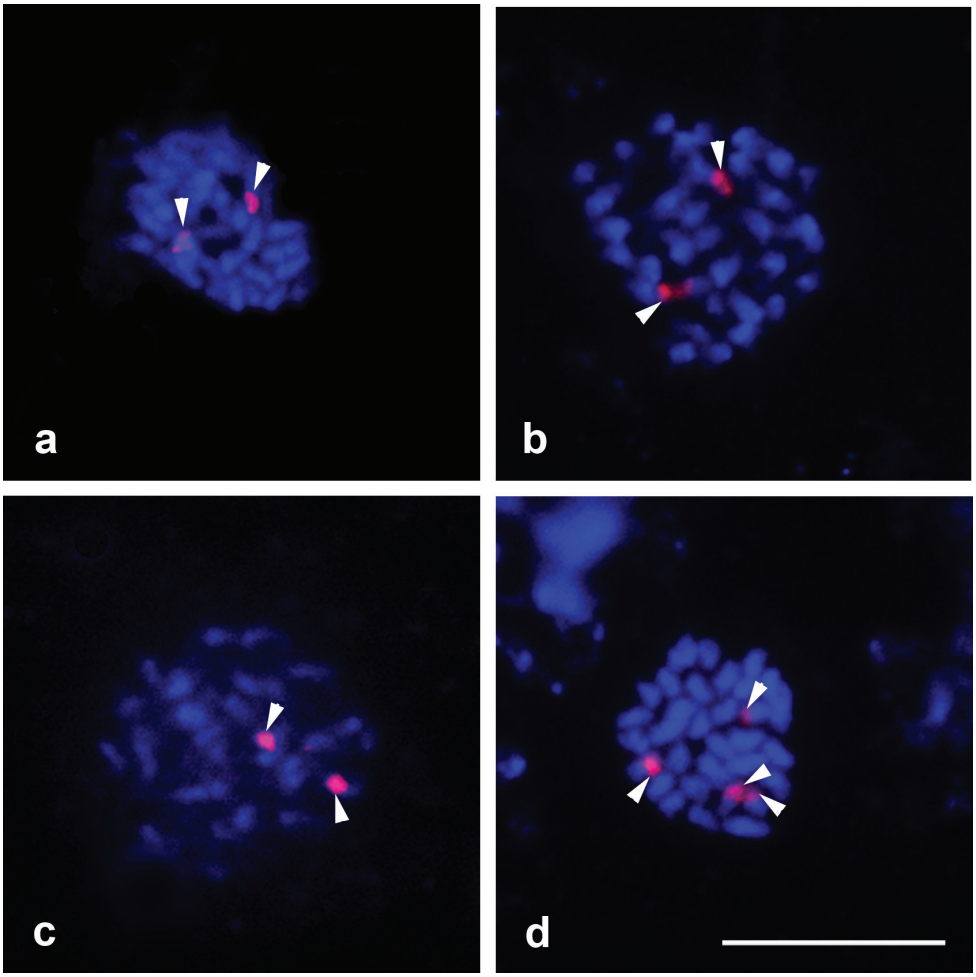

Figure 2. FISH with 18s ribosomal DNA **a** *Amaranthus cruentus* cv. Don Guiem **b** *A. hypochondriacus* cv. Artaza **c** *A. mantegazzianus* cv. Don Manuel **d** *A. caudatus* cv. EEA INTA Anguil the arrow indicates four signals. Bar = 5 µm.

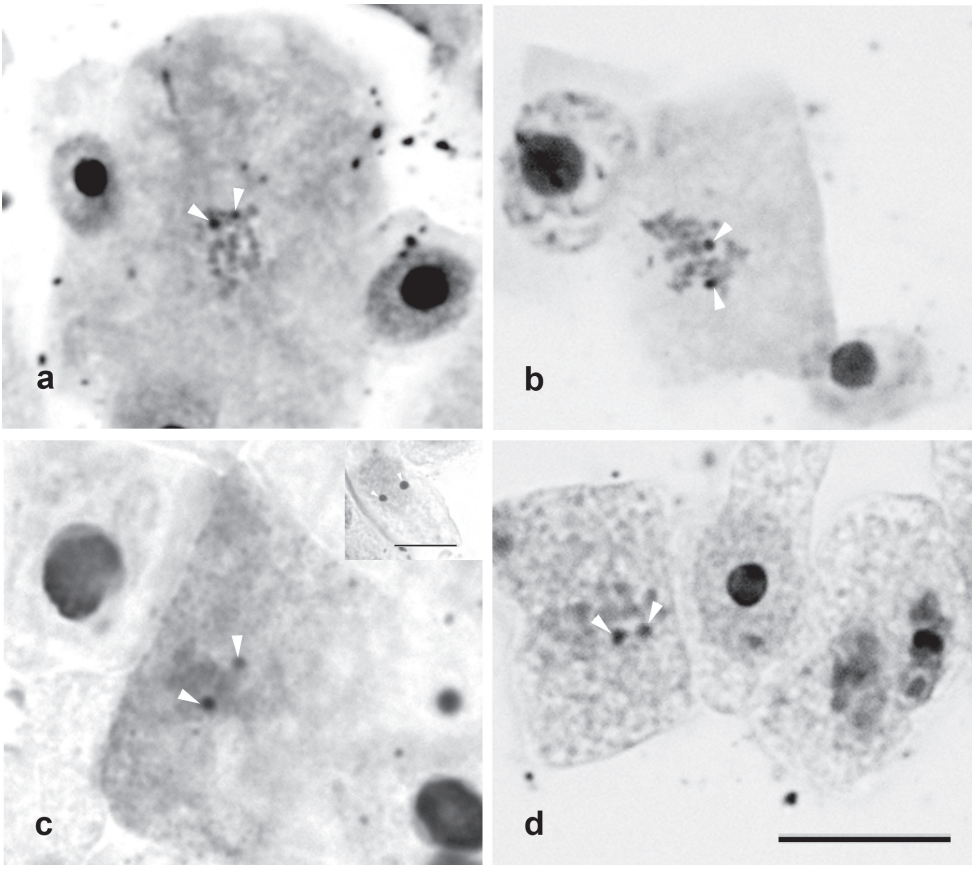

Figure 3. Ag-banding, **a** *Amaranthus cruentus* cv. INDEAR **b** *A. hypochondriacus* cv. Artaza **c** *A. mantegazzianus* cv. INDEAR and **d** *A. caudatus* cv. EEA INTA Anguil. Bar = 10 μm.

Ag-NOR technique (Fig. 3) allowed detection of one pair of chromosomes with active NOR in all studied materials. The results with FISH technique agree with the silver staining, which revealed two active NORs in the previous interphase. In the case of the four signals in *A. caudatus* cv. EEA INTA Anguil, only two would be coincident with active NORs.

Discussion

All the studied accessions of *Amaranthus hypochondriacus*, *A. mantegazzianus* and *A. caudatus* presented the chromosome number 2n = 32 and 2n = 34 for *A. cruentus,* which is in agreement with previous report (Greizerstein and Poggio 1994).

DAPI-CMA_3 banding showed that all the species and their cultivars had DAPI+ intersticials bands. In all accessions, two DAPI-/CMA_3+ bands were detected,

which were coincident with active NOR sites, even in the species *A. caudatus* which exhibited four of them. Nevertheless, differences among cultivars in the same species respect the number of DAPI+/CMA_3+ bands were found. For example, *A. cruentus* cv. INDEAR showed the higher number of these bands and *A. mantegazzianus* cv. Don Manuel did not present any. In the rest of the cultivars, two to six bands were observed, indicating the existence of inter and intraspecific differences in the quality and the amount of heterochromatin. Double staining with CMA_3 and DAPI is the combination most used to differentiate chromosome bands and for NOR identification (Guerra 2000). Some studies demonstrated NOR sites coincident with DAPI-/CMA_3+ bands (Guerra 2000; Barros e Silva and Guerra 2010). Due to CMA_3 marks the presence of rich in CG sequences, this technique not only highlights the NORs sites, but also other regions in the genome (Guerra 2000). According to this statement, Kolano et al. (2001) found that the amount of CMA_3+ bands in two other cultivars of *A. caudatus* could be more numerous than rDNA sites.

All studied cultivars of *A. cruentus*, *A. hypochondriacus* and *A. mantegazzianus* presented two hybridization signals by FISH. *Amaranthus caudatus* presented two hybridization signals in the cv. INDEAR, but four signals in cultivar EEA INTA Anguil. A similar result was detected, in the same species, by Kolano et al. (2001). They found one pair of signals in the cultivar Kiwicha 3, and two pairs in cv. Kiwicha Molinera, using FISH with ribosomal DNA probe. In the present work, four signals were detected in the cultivar EEA INTA Anguil, two of them were not coincident with Ag-NOR bands, which indicates the presence of inactive ribosomal loci.

In agreement with Greizerstein and Poggio (1994), we have detected a single pair of active NOR bands. The chromosomes carrying satellites, according these authors, were the fourth pair for *A. cruentus,* and the sixth pair for *A. hypochondriacus*, *A. mantegazzianus* and *A. caudatus*. These pairs of chromosomes could be the pair bearing the Ag-NOR bands, and they could be coincident with FISH results. However, Palomino and Rubí (1991) reported the presence of six to ten pairs of chromosomes with satellites. These differences could be showing a wide genetic variation masked by the few studied cultivars.

The difference in the amount of active NORs in the species *A. caudatus* cv. EEA INTA Anguil could be due to different sources. There are organisms which contain multiple NORs and many of them are silenced by epigenetic mechanisms. This silencing state of the NOR could be inherited by subsequent generations (McStay and Grummt 2008). The variation in the number of regions among cultivars could be due to this mechanism, or be a by-product of breeding programs.

To summarize, our results support the hypothesis that the cultivated *Amaranthus* species have two active NORs regions. Furthermore, the number of DAPI+/CMA_3+ bands allowed the characterization and identification of heterochromatin in cultivars and species. However, it would be interesting to study others cultivars and native populations.

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RESEARCH ARTICLE

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Comparative chromosomal analysis and evolutionary considerations concerning two species of genus *Tatia* (Siluriformes, Auchenipteridae)

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Abstract

Auchenipteridae is divided in two subfamilies, Centromochlinae and Auchenipterinae. Centromochlinae has 31 valid species, from which 13 are included in the genus *Tatia* Miranda Ribeiro, 1911. Among these, *Tatia jaracatia* Pavanelli & Bifi, 2009 and *T. neivai* (Ihering, 1930) are the only two representative species from the Paraná-Paraguay basins. This study aimed to analyze cytogenetically these two species and thus provide the first chromosomal data for the genus. Although *T. jaracatia* and *T. neivai* presented 2n=58 chromosomes, some differences were observed in the karyotypic formula. The heterochromatin was dispersed in the centromeric and terminal regions of most chromosomes of *T. jaracatia*, and only in the terminal region of most chromosomes of *T. neivai*. The AgNORs were detected in the subtelocentric pair 28 for both species, which was confirmed by FISH with 18S rDNA probe. The 5S rDNA sites were detected in four chromosome pairs in *T. jaracatia* and three chromosome pairs in *T. neivai*. Both species of *Tatia* presented great chromosomal similarities among themselves; however, when compared to other species of Auchenipteridae, it was possible to identify some differences in the karyotype macrostructure, in the heterochromatin distribution pattern and in the number and position of 5S rDNA sites, which until now seems to be intrinsic to the genus *Tatia*.

Keywords
Pericentric inversions, NORs, C-banding, 5S rDNA-FISH, 18S rDNA-FISH

Introduction

Among the Siluriformes, Auchenipteridae comprises a fish group endemic to the Neotropical region. The family comprises 20 genera and about 90 species (Ferraris Jr 2007), 74 of which have already been registered in Brazil (Akama and Sarmento-Soares 2007). According to Ferraris Jr (2003), Auchenipteridae is subdivided in two subfamilies, Centromochlinae and Auchenipterinae, which form monophyletic groups (Birindelli 2010). Most of the Auchenipteridae genera belong to the subfamily Auchenipterinae, with only *Centromochlus* Kner, 1857, *Gelanoglanis* Böhlke, 1980, *Tatia* Miranda Ribeiro, 1911 and *Glanidium* Lütken, 1874 allocated in Centromochlinae (Soares-Porto 1998). The subfamily Centromochlinae has 31 valid species (Ferraris Jr 2007), and in a revision of the genus, 12 species are described of *Tatia* (Sarmento-Soares and Martins-Pinheiro 2008). After this revision, a new species was described for this genus, *Tatia jaracatia* Pavanelli et Bifi, 2009, which is endemic to the Iguaçu River, a tributary of the Paraná River basin (Pavanelli and Bifi 2009).

The genus *Tatia* is found in the eastern region of the Andes, with wide distribution in South American drainages (Sarmento-Soares and Martins-Pinheiro 2008). Generally, fishes from this group are found in lentic environments of streams, rivers and lagoons and have nocturnal habits (Lowe-McConnell 1987). Most species can be found in rivers belonging to the Amazon River basin (Sarmento-Soares and Martins-Pinhiero 2008). The species studied in this paper [*T. neivai* (Ihering, 1930) and *T. jaracatia*] represent the only two species from the Paraná-Paraguay basins that belong to the genus *Tatia*, being *T. neivai* widely distributed in the Paraná and Paraguay basins and absent in the Iguaçu River basin.

Chromosomal analyses in Auchenipteridae are scarce and restricted to few species of the genera *Ageneiosus* La Cepède, 1803, *Auchenipterus* Bleeker, 1862, *Glanidium* and *Parauchenipterus* (Linnaeus, 1766). The two analyzed *Ageneiosus* species demonstrate diploid number of 56 chromosomes (Fenocchio and Bertollo 1992), while the other analyzed species [*Glanidium ribeiroi* Haseman, 1911, *Parauchenipterus galeatus* (Linnaeus, 1766) and *Auchenipterus osteomystax* (Miranda Ribeiro, 1918) cited as *A. nuchalis* (Spix et Agassiz, 1829)] have 58 chromosomes (Fenocchio and Bertollo 1992, Ravedutti and Júlio Jr 2001, Fenocchio et al. 2008, Lui et al. 2009, Lui et al. 2010). Until now, there were no chromosomal studies in *Tatia* species. Therefore, this study aimed to cytogenetically analyze the two species of the Paraná-Paraguay drainage belonging to the genus *Tatia* (*T. neivai* and *T. jaracatia*), generate the first chromosomal data concerning the genus and thus allowing differentiation of closely related species.

Material and methods

Chromosomal analysis was performed on 17 specimens (15 males and 2 females) of *T. neivai* from Machado River, a tributary of the Bugres River, Paraguay River basin, Denise city, Mato Grosso, Brazil (14° 40'43"S, 57°00'47"W), and 10 specimens (7 males and 3 females) of *T. jaracatia* from the Iguaçu River basin, Capanema city, Paraná, Brazil (25°35'19"S, 53°54'48"W). The specimens were deposited in the fish collection of Museum of Zoology of University of São Paulo (*T. jaracatia*, MZUSP 109792; *T. neivai*, MZUSP 109794).

Specimens were previously treated with 0.05% colchicine solution (1 ml/100 g body weight), 30-40 minutes before sacrifice, and the cell suspension of mitotic chromosomes was obtained from the anterior kidney cells (Bertollo et al. 1978, Foresti et al. 1993). Thirty metaphase plates from each fish were examined and 10 of the best mitotic metaphases were used to measure karyotypes. Chromosome morphology was determined according to Levan et al. (1964). The fundamental number (NF) was calculated considering metacentric (m), submetacentric (sm) and subtelocentric (st) chromosomes as having two arms, and acrocentric chromosomes (a) as having only one arm. The heterochromatic pattern was determined according to Sumner (1972) with modifications in the staining process (Lui et al. 2012), and the nucleolus organizer regions (NORs) were identified using silver nitrate impregnation (Howell and Black 1980). Both methods were administered sequentially, following the conventional chromosome staining with Giemsa (sequential analysis).

The fluorescence *in situ* hybridization (FISH) was performed according to Pinkel et al. (1986). The 5S and 18S rDNA probes were obtained according to Martins et al. (2000) and Hatanaka and Galetti Jr (2004), respectively. The 5S and 18S rDNA probes were labeled by nick translation with biotin-16-dUTP and digoxigenin-11-dUTP (Roche), respectively. Probes labeled with biotin were detected and amplified with avidin-FITC and anti-avidin-biotin (Sigma). The other probes labeled with digoxigenin were detected with anti-digoxigenin-rhodamine (Roche). Chromosomes were counterstained with DAPI solution and analyzed in the epifluorescence microscope Olympus BX50. Images were captured with the DP2-BSW software (Olympus).

Results

Tatia jaracatia

Cytogenetical analysis revealed the diploid number of 58 chromosomes (20m+26sm+12st, FN=116) (Fig. 1a). The heterochromatin presented itself disperses in the centromeric and terminal regions of most chromosomes of the karyotype (Fig. 1b). The silver nitrate impregnation showed only the subtelocentric pair 28 marked in the terminal position

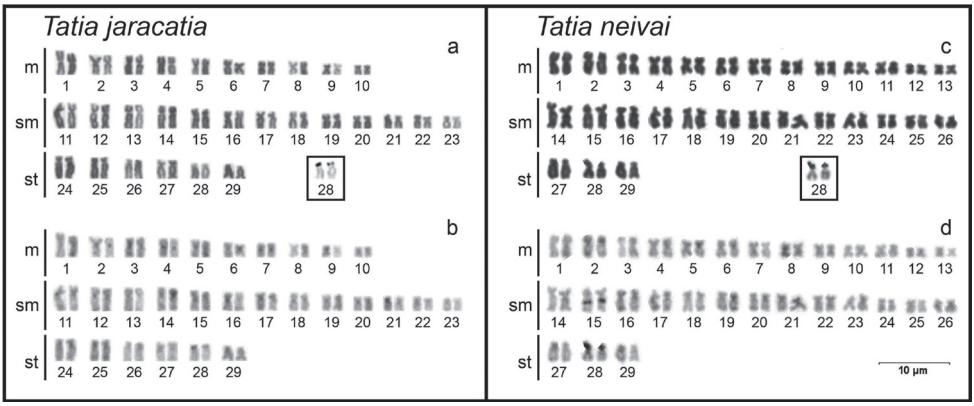

Figure 1. Karyotypes of *Tatia jaracatia* (**a, b**) and *Tatia neivai* (**c, d**) stained with Giemsa (**a, c**) and sequentially C-banded (**b, d**). The AgNORs bearing chromosomal pair is presented in box.

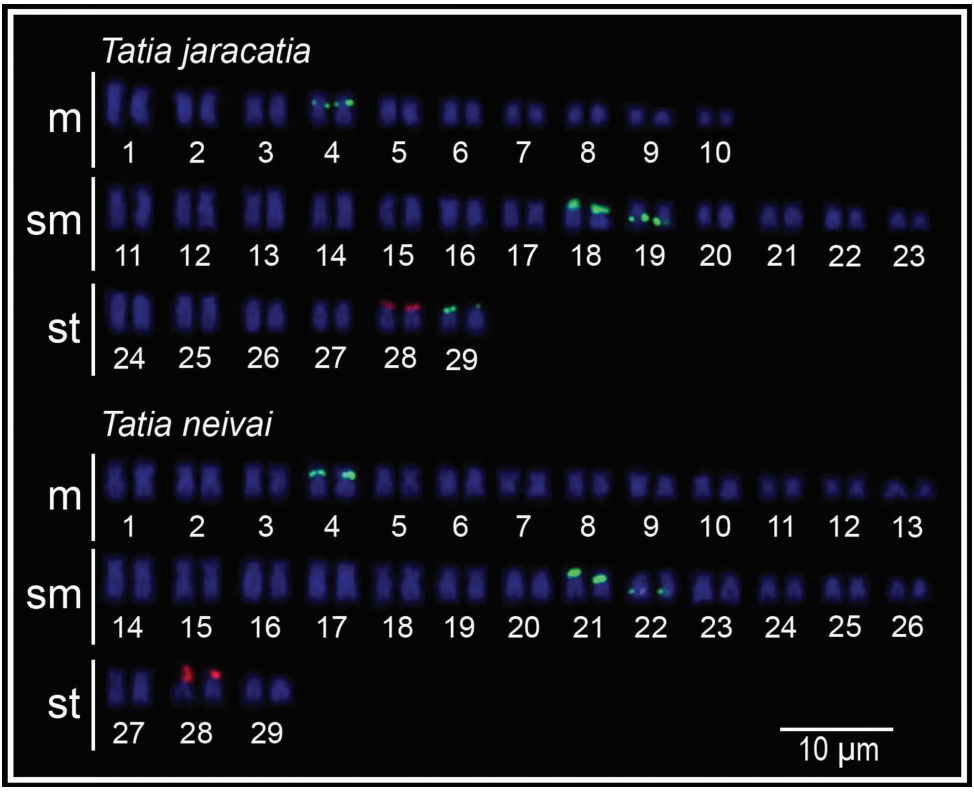

Figure 2. Karyotypes of *Tatia jaracatia* and *Tatia neivai* 5S rDNA-FISH (FITC, green) and 18S rDNA-FISH (digoxigenin, red).

of the short arm (Fig. 1a, in box). FISH with 18S rDNA probe showed only one labeled chromosome pair (pair 28) corresponding to the silver nitrate impregnation. The 5S rDNA sites were detected in 4 chromosome pairs (pairs 4, 18, 19 and 29), on the short

arm in interstitial position of the metacentric pair 4, on the short arm in terminal position of the submetacentric pairs 18 and 29, and on the long arm in interstitial position of the submetacentric pair 19 (Fig. 2).

Tatia neivai

Cytogenetical analysis revealed the diploid number of 58 chromosomes (26m+26sm+6st, FN=116) (Fig. 1c). The heterochromatin showed itself poorly marked and dispersed in the terminal region of most chromosomes of the karyotype, with the exception of two conspicuous blocks: one in interstitial position on the long arm of submetacentric pair 15, and other in terminal position on the short arm of subtelocentric pair 28 (Fig. 1d), corresponding to the NORs (Fig. 1c, in box). FISH with 18S rDNA probe showed only one labeled chromosome pair, the subtelocentric pair 28, corresponding with the silver nitrate impregnation. The 5S rDNA sites were detected in 3 chromosome pairs (pairs 4, 21 and 22), being in the interstitial position of the short arm of metacentric pair 4, in terminal position of the short arm of submetacentric pair 21, and in interstitial position of the long arm of submetacentric pair 22 (Fig. 2).

No intraspecific polymorphism related to diploid number, karyotypic formula, C banding, 5S and 18S rDNA (including AgNORs) were observed in both species.

Discussion

Chromosomal studies in Auchenipteridae have shown that most analyzed species have diploid number of 58 chromosomes (Ravedutti and Júlio Jr 2001, Fenocchio et al. 2008, Lui et al. 2009, Lui et al. 2010), with the exception of species from the *Ageneiosus* genus that have 56 chromosomes (Fenocchio and Bertollo 1992). The genus *Tatia* is included in the subfamily Centromochlinae, which had only one species with chromosomal analysis to date, *Glanidium ribeiroi*, which also has 2n=58 chromosomes, as well as the two species of *Tatia* analyzed in this paper. The 2n=58 chromosomes is shared by species of the *Auchenipterus* and *Parauchenipterus* genera (subfamily Auchenipterinae), and 14 out of the 16 already analyzed species of the Doradidae family (Eler et al. 2007, Milhomem et al. 2008), which is considered sister-group of Auchenipteridae (Pinna 1998). Thus, it is likely that 2n=58 chromosomes is basal, not only in Auchenipteridae, but also in Centromochlinae. This hypothesis is reinforced by the fact that 2n=58 chromosomes is considered basal for Doradidae (Milhomem et al. 2008).

The fundamental number (FN=116) found for the two *Tatia* species in this paper is higher than found in other Auchenipteridae species studied so far. This difference is due to an increase in the number of chromosomes bearing two arms in the detriment of chromosomes carrying only one arm (Fig. 1a, c). This absence of acrocentric chromosomes was not detected in other species of the family yet, and seems to be an intrinsic characteristic of the genus *Tatia*, or at least of a specific clade formed by the

species studied here. Thus, considering the maintenance of the diploid number, the variations in the karyotypic formula and FN of analyzed species, when compared with other species from others Auchenipteridae genus, it is evident that non-Robertsonian rearrangements, here represented by pericentric inversions, must be active mechanisms in the karyotypic diversification of *Tatia* species.

The heterochromatin distribution pattern found in *T. jaracatia* and *T. neivai* differs in some aspects from other Auchenipteridae species. Besides heterochromatic blocks in the terminal region of chromosomes, which are commonly found in most Auchenipteridae species, heterochromatin sites were observed in the centromeric region of some chromosomes in *T. jaracatia*, and a conspicuous block in the interstitial region of the submetacentric pair 15 of *T. neivai* (Fig. 1b, d). No interstitial heterochromatin blocks were detected in *T. jaracatia*.

The silver nitrate impregnation had only one subtelocentric chromosome pair marked on the short arm in terminal position (pair 28) in both species (Fig. 1, in box), as confirmed by FISH with 18S rDNA probe (Fig. 2). This pair is likely correspondent between species. According to Ravedutti and Júlio Jr (2001), simple NORs in interstitial position seem to be a characteristic of Auchenipteridae. In Doradidae (sister-group), NORs vary in number and type of the bearing chromosome pairs among the 16 species studied so far (Milhomem et al. 2008). According to the same authors, pericentric and paracentric inversions may have acted in the karyotype evolution of the group changing the location of these sites. A similar situation may have occurred in Auchenipteridae. Although there are slight variations in the location of these sites among the species of this family, it is likely that the 18S rDNA bearing chromosome pairs are corresponding among them. Given this context, the fact that the NORs are always located on a single chromosome pair may suggest this condition is a putative basal character of the clade composed by the Auchenipteridae and Doradidae families. Regarding the *Tatia* species, the data for this chromosome pair also suggest a conserved status for the genus because of the location and type of the chromosome pair bearing these genes.

The data of 5S rDNA sites physical mapping by FISH in Auchenipteridae are scarce and only refer to *P. galeatus* populations (Lui et al. 2010), which presented sites located in interstitial position of two submetacentric pairs: one pair on the short arm and another on the long arm, which change in location in the karyotype among populations of this species. In both species of *Tatia*, two submetacentric pairs (pairs 18 and 19 in *T. jaracatia*; pairs 21 and 22 in *T. neivai*) bearing the 5S rDNA cistrons were observed, with location similar to the two 5S rDNA bearing chromosome pairs in the different populations of *P. galeatus* (Lui et al. 2010). It is likely that due to the similar morphology and location, these pairs may be considered correspondent among the species, even though they present great phylogenetic distance within the family. The metacentric pair 4 shows 5S rDNA sites in interstitial position in the short arm, which is shared by both *Tatia* species. The 5S rDNA cistrons of pair 4 and the other two aforementioned can be considered as matching between *T. jaracatia* and *T. neivai*; however, the site present in terminal position on the short arm of the subtelocentric

pair 29 seems to be a unique feature of *T. jaracatia*. Despite the multiple conditions with more than two pairs bearing the 5S rDNA cistrons being shared by many species of Auchenipteridae, this marker appears to present greater diversity in the family Auchenipteridae when compared to other commonly used markers.

According to the phylogeny of Soares-Porto (1998), *T. neivai* is sister-group of *T. bohemia* Koch et Reis, 1996, being the latter found only in the Uruguay River, and with *T. jaracatia*, these three species are the only valid species for the La Plata basin (Uruguay, Paraguay and Paraná Rivers). Thus, despite the great geographic distance that separates the species analyzed in this paper, it is possible to assume that *T. neivai* and *T. jaracatia* present significant phylogenetic proximity, which explains the great similarity found with most of the markers. However, when comparing both *Tatia* species from this paper with other species of Auchenipteridae, we can observe that the karyotypic formula (mainly due to the lack of acrocentric chromosomes) and the distribution pattern of the heterochromatin and 5S rDNA sites differ from the rest of the group showing some characteristics which until now appear to be intrinsic to the genus *Tatia*.

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RESEARCH ARTICLE

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Experimental hybridization and chromosome pairing in *Kosteletzkya* (Malvaceae, Malvoideae, Hibisceae), and possible implications for phylogeny and phytogeography in the genus

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Abstract

Kosteletzkya C. Presl, 1835 (Malvaceae, Malvoideae, Hibisceae) includes 17 species, all but two of which are about evenly distributed between Africa and the northern Neotropics. Fifteen of the species were brought into cultivation and used in a hybridization program in an attempt to shed light on evolutionary and phytogeographic relationships in the genus. Chromosome pairing (x = 19) at meiosis was examined in 51 of the 56 interspecific hybrids that were produced, and the seven New World species, all diploids, were found to exhibit nearly complete pairing among themselves, indicating that they share a genome. By contrast the three African diploids showed low levels of chromosome pairing in crosses among themselves, leading to the recognition here of three distinct genomes, newly designated A, B and G. The African B-genome diploid, *K. buettneri* Gürke, 1889, was found to share its genome with the New World species. Four other African species are known to be tetraploids and a fifth, a hexaploid. The results of chromosome pairing in hybrids among all of the African species at all ploidy levels, plus the discovery of a spontaneously tetraploidized experimental intergenomic African diploid hybrid, suggest that three of the four tetraploids and the single hexaploid might all be allopolyploids built on the three known extant genomes. The fourth tetraploid paired poorly or moderately with these three genomes. Results are consistent with the hypothesis that *Kosteletzkya* arose in Africa, radiated at the diploid level, underwent natural interspecific hybridization, produced two tiers of allopolyploids, and at some more recent time dispersed a B-genome diploid to the New World where it underwent another radiation at the diploid level. Structural features of the fruits suggest adaptations for passive distribution by animals, potentially over long distances.

Keywords
Kosteletzkya, Hibisceae, Malvoideae, Malvaceae, hybridization, chromosomes, phytogeography, phylogeny

Introduction

Kosteletzkya C. Presl, 1835 (Malvaceae, Malvoideae, Hibisceae) comprises 17 species that, with two exceptions, are about evenly divided between Africa (eight species) and the northern New-World tropics (seven species; Blanchard 2012). One of the exceptions, *K. pentacarpos* (Linnaeus, 1753) Ledebour, 1841, is found primarily extratropically along the eastern and Gulf coasts of the United States, with a few probably introduced populations in Eurasia (Blanchard 2012); the other exception, *K. batacensis* (Blanco, 1837) Fernández-Villar, 1880, is found only on the island of Luzon in the Philippines (Borssum-Waalkes 1966). At present, *Kosteletzkya* sits awkwardly within the paraphyletic genus *Hibiscus* Linnaeus, 1753 along with *Pavonia* Cavanilles, 1786, *Abelmoschus* Medikus, 1787, *Taliparitì* Fryxell, 2001, *Wercklea* Pittier & Standley, 1916, and several other, mostly smaller genera (Pfeil and Crisp 2005), but *Kosteletzkya* itself is well circumscribed (Blanchard in Verdcourt and Mwachala 2009). Structurally the genus is distinctive among the Hibisceae in that its 5-valved, 5-angled or -winged capsules contain a single seed per locule, and the valves themselves ultimately separate both from one another and from the fruiting axis. This characteristic of fruit disintegration, along with other features, excludes several endemic Madagascan species that are generally placed in *Kosteletzkya*, but which clearly belong elsewhere. Recent DNA evidence supports this interpretation (Koopman and Baum 2008).

The species of *Kosteletzkya* are mostly herbaceous perennials that bear small to medium-sized Hibiscus-like flowers (Figs 1, 6A–P) that usually last for a single day. Indigenous uses have been reported for several of the species (Chevalier 1940, Iljin 1949, Morton 1981, Anokbonggo et al. 1990, Burkill 1997), but only the temperate *Kosteletzkya pentacarpos* has received much attention for its more general economic potential (see Halchak et al. 2011). On account of its salt tolerance (Somers 1978, Grant and Somers 1981, Gallagher 1985, Blits et al. 1993, Poljakoff-Mayber et al. 1994) the plant can be grown as a crop on otherwise non-arable soil, and this has led in turn to studies that have identified *K. pentacarpos* as a potential commercial fiber source and have also shown that the seeds may be harvested as potential sources of biodiesel fuel and animal feed (Nekrasova and Pankova 1949, Islam et al. 1982, Ruan et al. 2008b). The same species has also found minor commercial use in the horticultural trade, especially for native-plant gardens.

The base chromosome number in *Kosteletzkya* is 19, and counts have been reported for 15 of the 17 species (Blanchard 1974, 2012; Table 1). The seven New-World species, with a center of diversity in Mexico, are all diploids. By contrast, the eight African species include three widely distributed diploids and, with more restricted distributions, four tetraploids (including the newly described *K. rotundalata* O. J. Blanchard,

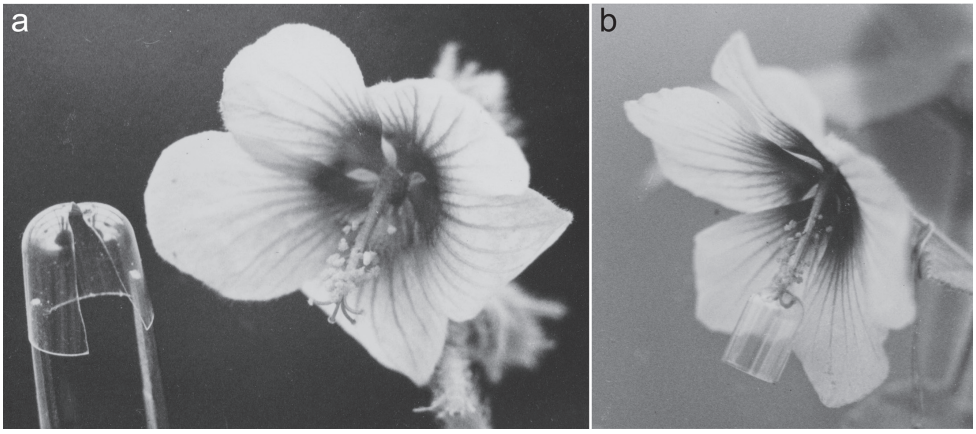

Figure 1. Simple gelatin-capsule device for preventing self-pollination in experimentally manipulated flowers of *Kosteletzkya*. **a** Perforated capsule-half spread with forceps in preparation for placement around the base of the style-branches of a flower of *K. begoniifolia* (76-1) **b** Capsule-half closed and in place between the base of the style-branches and the pollen mass. Note the recurved styles pressing their stigmas against the inside of the capsule-half.

Table 1. Chromosome numbers in *Kosteletzkya* (x = 19). Data from Blanchard 1974, 2012.

New World Species	Chromosome number (n)	African Species	Chromosome number (n)
K. blanchardii Fryxell, 1977	19	*K. adoensis* (A. Richard, 1847) Masters, 1868	19
K. depressa (Linnaeus, 1753) O. J. Blanchard, Fryxell et D. M. Bates, 1978	19	*K. buettneri* Gürke, 1889	19
K. hispidula (Sprengel, 1815) Garcke, 1881	19	*K. grantii* (Masters, 1868) Garcke, 1880	19
K. pentacarpos (Linnaeus, 1753) Ledebour, 1841	19	*K. begoniifolia* (Ulbrich, 1917) Ulbrich, 1924	38
K. ramosa Fryxell, 1977	19	*K. borkouana* Quézel, 1957	38
K. reclinata Fryxell, 1977	19	*K. rotundalata* O. J. Blanchard, 2013	38
K. tubiflora (de Candolle, 1824) O. J. Blanchard et McVaugh, 1978	19	*K. semota* O. J. Blanchard, 2008	37–38
		K. racemosa Hauman, 1961	57

2013 [Blanchard 2013]), and one hexaploid. Meiotic figures of representative diploid, tetraploid and hexaploid species are shown in Figs 2a, 2c and 3a.

The bi-centric geographical distribution of *Kosteletzkya* raises a question of where the group originated. Because the more complex, polyploid-rich species assemblage in Africa suggests a longer evolutionary history than its uniformly diploid New-World counterparts, I have speculated that Africa was the birthplace of the genus (Blanchard 2012).

Elsewhere in the Malvoideae, interspecific hybridization trials and the study of chromosome behavior in the resulting hybrids have been useful in clarifying species affinities, phytogeography and genomic differentiation. The two best-documented ex-

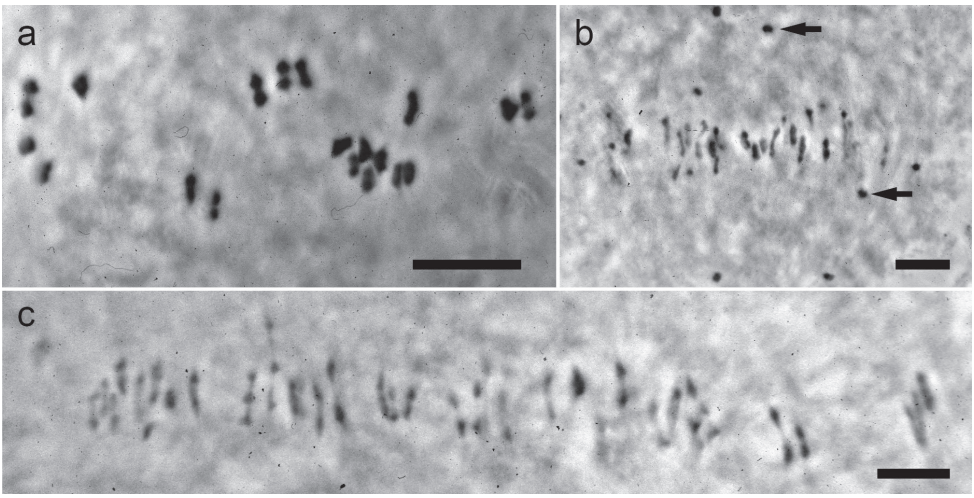

Figure 2. Phase-contrast photographs of meiotic metaphase I figures in two *Kosteletzkya* species and a tetraploid-tetraploid interspecific hybrid. **a** *K. adoensis* (76-36), 19_{II} **b** *K. borkouana* × *K. begoniifolia* (77-142), $22_{II} + 32_{I}$ **c** *K. borkouana* (76-40), 38_{II}. Arrows in b. indicate two of the 22 univalents. Scale bar = 10 μm.

amples are the cotton genus *Gossypium* Linnaeus, 1753 and *Hibiscus* sect. *Furcaria* de Candolle, 1824. Each includes both diploid and polyploid species and each is distributed on both sides of the Atlantic, as well as in Australia. *Gossypium* comprises about 50 species (Fryxell in Verdcourt and Mwachala 2009) and eight distinct genomes (Fryxell 1992, Cronn and Wendel 2004), while *Hibiscus* sect. *Furcaria* boasts over 100 species and 13 identified genomes (Krapovickas 2006, Wilson 2006). With a few intriguing exceptions their genomes correspond to, or are confined to, distinct geographical areas.

Over a period of several years I have accumulated a living greenhouse collection of 15 *Kosteletzkya* species, and during that time I have incorporated them into a hybridization program that has attempted to shed light on the phytogeography and evolutionary history of the genus. The results are presented here. This information is expected in turn to illuminate molecular-level investigations of *Kosteletzkya* currently being pursued by the author and colleagues at the University of Florida.

Materials and methods

Table 2 shows the greenhouse numbers, provenances and collectors of the 31 living accessions (i.e. cultivated progeny from a single seed source) of the 15 species used in this study, as well as six additional accessions from which the flowers in Fig. 6 were photographed.

Plants of *Kosteletzkya* grow readily under glass. Uniform germination was obtained by chipping away a bit of the seed coat at the radical end of the seed. When seeds were started in the spring, these mostly short-day plants came into flower in the following fall and winter.

Table 2. Sources of 38 greenhouse-grown accessions of *Kosteletzkya*. Thirty-two accessions, representing 15 species plus an artificial tetraploid, were used in the hybridization work. Six others were sources of some of the flowers photographed for Fig. 6. Species names are followed by one or more bolded greenhouse numbers in which the year of cultivation is indicated by the two digits preceding the hyphen.

***K. adoensis*, 74-22,** ANGOLA: Huambo Distr., *Instituto de Investigação Agronómica de Angola s.n.*; **75-191,** SIERRA LEONE: Loma Mountains, on plateau at camp 2, *Morton SL 418*; **76-36,** ETHIOPIA: Caffa a Bonga, *Saccardo 40*; **10-14**, ETHIOPIA: Gonder: Libo Awraja, ca. 15 km N of Addis Zemen, *Tadesse and Kagnew 1973*; **80-107,** MALAWI: N. Prov.: Nkhata Bay Dist.: 5 mi E of Mzuzu, *Pawek 11872*; **10-31,** MALAWI: Nkhata Bay Dist.: Vipya Plateau, 23 mi SW of Mzuzu, *Pawek 11275*;

***K. "art,"* 80-103, 82-90,** an artificial tetraploid, i.e. a spontaneously tetraploidized plant derived from an artificial hybrid between the two greenhouse plants *K. adoensis* 75-191 and *K. grantii* 76-2;

***K. begoniifolia*, 76-1, 10-64,** TANZANIA: Lerai Forest, Ngorongoro Crater, *Bonnefille and Riollet 73/26*; **79-21,** ETHIOPIA: ca. 40 km W of Ambo, *de Wilde and de Wilde-Duyfjes 10421*; **79-53,** KENYA: Seboti, SE Elgon, *Tweedie 3242*;

***K. blanchardii*, 74-10, 76-21, 88-10, 90-19, 10-26,** MEXICO: Michoacán, 13 mi N of Tuzantla, *Fryxell, Bates and Blanchard 1650*;

***K. borkouana*, 76-40, 10-21,** UGANDA: "Rhino Camp," Bahr el Jebel, Lado Enclave, *Mearns 2803*; **79-44,** CHAD: Borkou, Tigui, *Quézel s.n.*; **79-31, 90-36,** CONGO-KINSHASA: Plaine de la Ruzizi, Lac Tsimuka, *Germain 5682*;

***K. buettneri*, 74-11, 76-22, 88-6**, ZAMBIA: "C Province," Kafue Pontoon, *Robinson 6706*; **90-27,** ZAMBIA: Chingola, *Handlos s.n.*; **90-25, 10-59,** TANZANIA: Buha Dist., Malagarasi Ferry, 40 mi. from Kibondo on Kasulu road, *Verdcourt 3444*; **79-28, 88-18,** CONGO-KINSHASA: Kipopo, près d'Elisabethville (Katanga), *Symoens 9242*; **90-8,** MALAWI: Bua River below Mude River confluence, *Robson 1542;*

***K. depressa*, 74-9, 75-136, 88-9, 90-17**, MEXICO: Nayarit: 30 mi. S of Compostela, *Fryxell, Bates and Blanchard 1563*; **10-18**, MEXICO: Sinaloa: between Rosario and Esquinapa, *Gentry, Barclay and Arguelles 19464*;

***K. grantii*, 76-2,** CONGO-KINSHASA: Dungu, *Gérard 758*; **79-41, 89-71,** KENYA: Between Sio ["Soi"] River and Busia, *Evans and Erens 1655*; **10-104**, NIGERIA: Zaria: Jemaa, Sanga River Forest Reserve, *Keay 37217*;

***K. hispidula*, 74-6, 88-23, 90-39**, MEXICO: Sinaloa: S of Mazatlán, *Fryxell and Bates s.n.*; **10-44**, MEXICO: Sonora: N of El Sahuaral, *Felger and Reichenbacher 85-1581*;

***K. pentacarpos*, 74-19,** USA: Florida: Seminole Co., Lake Monroe N of Sanford, *Blanchard and Blanchard 306*; **88-8,** USA: Virginia: Chesterfield Co., N of Bermuda Hundred, *Harvill 17659*; **74-15,** USA: Florida: Sarasota Co., Laurel, *Blanchard and Blanchard 302*; **10-81,** IRAN: Astara ["Astava"], *Wright 62*; **80-142**, an intraspecific hybrid between the following two greenhouse plants**:** 79-16, USA: Louisiana: Cameron Parish, Hackberry, *Blanchard and Blanchard 423,* and 79-38**,** IRAN: Astara ["Astava"], *Wright 62*;

***K. racemosa*, 79-24, 82-88, 90-5, 10-45,** CONGO-KINSHASA: Gandajika, *Liben 3266*;

***K. ramosa*, 88-1**, **10-23**, MEXICO: Jalisco: 1 mi E of Ayotlán, *Blanchard and Blanchard 1148*;

***K. reclinata*, 88-15, 10-5,** MEXICO: Jalisco: 11.7 km W of Tototlán, *Blanchard and Blanchard 1149*;

***K. rotundalata*, 80-104, 90-20, 10-53**, CONGO-KINSHASA: Nizi, *Liben 444*;

***K. semota*, 90-2, 10-100,** NIGERIA: Ogun, Omi R., Ogun Makin, *Daramola s.n.*;

***K. tubiflora*, 74-24, 90-11, 10-22,** MEXICO: Jalisco: NE of Guadalajara, Barranca de los Oblatos, *Fryxell, Bates and Blanchard 1590*; **76-23, 78-14**, MEXICO: Jalisco: K22 W of Guadalajara, *Fryxell and Bates 2137*.

Cross-pollinations

In most species of *Kosteletzkya*, a flower persists for only a single day; by early afternoon it has already begun to wither. Cross-pollinations were therefore performed by hand in the morning, shortly after the flowers had fully opened. As has been reported for *K. pentacarpos* (Ruan et al. 2008a, 2011), most species of *Kosteletzkya* recurve their styles later in the day and push their stigmas into the pollen mass, thereby effecting self-pollination in the absence of any earlier exogenous pollination. This creates a problem when controlled crosses are attempted because it is impossible to be certain that successful seed-set was due to pollen from the other experimental parent rather than from the same flower. In the case of the larger, sturdier flowers found in some other Malvoideae, the pre-anthesis removal of the anthers solves this problem. This has been done, for example, in *Hibiscus* sect. *Furcaria* (Menzel and Wilson 1961), *Hibiscus* sect. *Muenchhusia* (Fabricius, 1763) O. J. Blanchard, 1988 (Wise and Menzel 1971), and *Gossypium* (Wilson and Stapp 1985). However the same technique was found to be too traumatic and inefficient for smaller flowers such as those of *Kosteletzkya*.

A solution to this problem was found in the use of halves of gelatin capsules to separate the male and female parts of the flowers (Fig. 1a). In this technique a hole is punctured through the apex of a capsule-half using a dissecting needle that has been heated in a flame. A razor blade is then used to cut up one side of the capsule-half wall and over the top, passing through the previously cut hole. It is then possible to 1) insert the closed tines of a straight forceps into the open end of the capsule-half, 2) allow the tines to spread the razor-cut slit, 3) slip the whole unit over the base of the style branches distal to the anther mass, and then 4) allow the capsule-half to close, effectively isolating the stigmas and pollen mass from one another (Fig. 1b). In the present study each such unit was attached to a flower immediately after a cross-pollination, and the procedure proved to be highly successful in preventing self-pollination. The only drawback was that extra care had to be taken when watering the plants in order to avoid deforming or dissolving the capsule-halves.

The gelatin-capsule device could not be used with one of the species. *Kosteletzkya borkouana* Quézel, 1957 is effectively an obligate selfer because the stigmas have usually already recurved into the pollen mass by the time the corolla opens in the morning. To make matters more difficult, this species has the smallest flowers of any *Kosteletzkya.* Nevertheless it was necessary to visit the plants at 0300-0400h to carefully cut away the unopened corolla and remove the mercifully few pre-dehiscence anthers. And because the time was still hours away from the anthesis of any of the other species, actual manual cross-pollination of the emasculated flower had to await a later visit.

While most of the species that were studied were short-day, fall-and-winter-flowering plants, three of them flowered in the late summer (*K. pentacarpos)* or early fall (*K. ramosa* Fryxell, 1977 and *K. reclinata* Fryxell, 1977). To make these three available

to a greater variety of other potential crossing partners, beginning in early summer plants of several of the other species were put on carts, moved daily at 1700h into an adjoining darkened room, and retrieved the next morning. Within three or four weeks, flower-bud initiation was evident. Early flowering was thereby induced so as to coincide with the flowering of the three late-summer-early-fall species.

In general, crosses worked in both directions. It appeared to make no difference in the success of an attempted cross whether a participant in the cross was the ovule-parent or pollen-parent, so no tabulated distinction is made here concerning the direction of the crosses reported. As a matter of insurance, however, the actual practice was that whenever two plants were crossed in which the size of the flowers, or more especially the style lengths, were considerably different, the smaller of the pair was used as the pollen recipient, on the theory that pollen adapted to traversing a short style might be challenged by a longer style (Williams and Rouse 1990, Sorensson and Brewbaker 1994, Tiffin et al. 2001).

Voucher specimens of most of the plants used as parents of crosses in this study, as well as specimens of the hybrids themselves, are deposited at the University of Florida Herbarium (FLAS), Florida Museum of Natural History, Gainesville, Florida, USA. In a few cases the vouchers for the parents are either the original wild-collected specimens that were the seed sources for the greenhouse plants, or they are specimens from the same seed source but grown in other years.

Pollen stainability

Pollen was stained with Cotton Blue in lactophenol. Each pollen slide was made from a single flower. Normal-sized, fully and deeply blue-stained grains were treated as "stained" and are expressed here as a percent of the total number of grains on a slide. At least three and usually five or more slides were counted for each hybrid combination. The majority of the species that participated as parents in the stainability evaluations were themselves counted and their stainabilities were found to range from 97 to 100 percent. Later in the hybridization program space was at a premium and the few replicates of hybrid plants that could be grown were used almost solely as a source of young flower buds for meiotic samples, so for some of the later-produced hybrids no fruit or pollen data were obtained. By that time, however, the general patterns of fruit-set and pollen stainability were already evident.

Fruit-set

Recurvature of the styles is a problem for controlled pollinations, but it is a boon for fruit-set purposes because, with one exception, it was theoretically possible to let the greenhouse hybrids pollinate themselves and use those results rather than resorting to manual self-pollination. In actual practice, however, the plants were usually hand-pollinated anyway, as a part of the routine of nearly daily visits to the greenhouse.

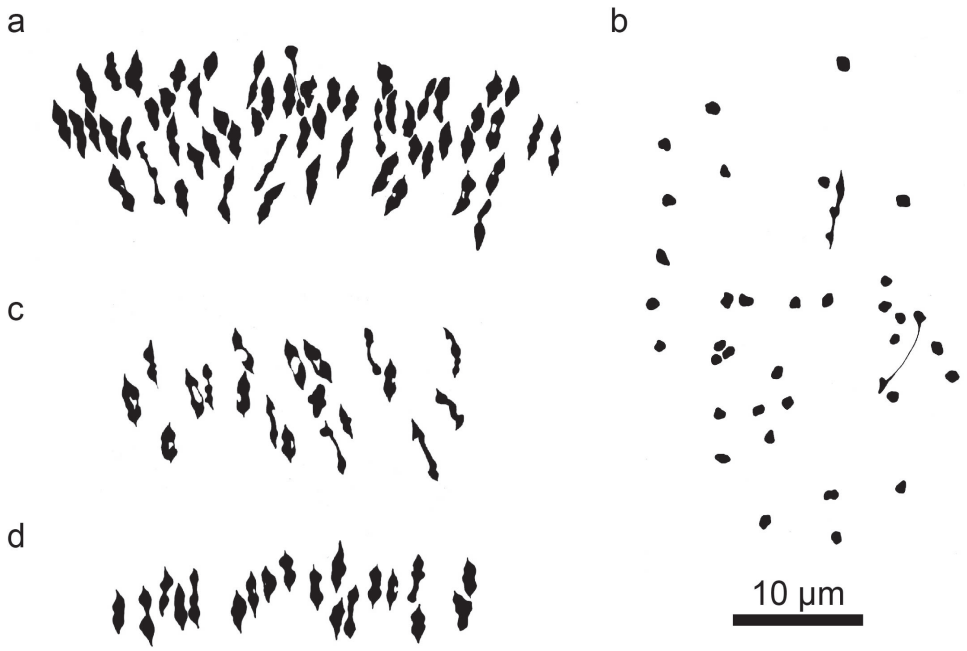

Figure 3. Camera lucida drawings of meiotic metaphase I figures in a species of *Kosteletzkya* and three diploid interspecific hybrids. **a** *K. racemosa* (82-88), 57_{II} **b** *K. depressa* × *K. adoensis* (77-136), $2_{II}+34_{I}$ **c** *K. depressa* × *K. tubiflora* (75-149), 19_{II} **d** *K. buettneri* × *K. hispidula* (Sprengel, 1815) Garcke, 1881 (75-180), 19_{II}.

The exception is hybrid progeny in which *K. tubiflora* (de Candolle, 1824) O. J. Blanchard & McVaugh, 1978 is one parent. This species bears distinctive yellow-and-red tubular flowers with an exserted staminal column and style (Figure 6G), and they are almost certainly bird-pollinated in the plant's native setting. The related *K. thurberi* A. Gray, 1887 with structurally similar flowers, was reported to be visited by the Bumblebee Hummingbird (*Atthis heloisa* [Lesson and DeLattre, 1839]; reported as "*Selasphorus heloisa*") in northern Mexico (Van Devender et al. 2004). Unlike the rest of the species, *K. tubiflora* has protogynous flowers that remain open and nectar-producing for two days or more. On the first day the stigmas are receptive and the anthers remain undehisced. By the next day the staminal column has elongated, exserting the now-dehiscent anthers to the earlier position of the stigmas. At this point, the stigmas may or may not remain receptive, but they do not recurve in the absence of pollination. As a consequence, the hybrids involving this species required hand-pollination.

Fruits will set in *Kosteletzkya* when as few as one of the five ovules has been fertilized, and no distinction is made here as to the number of seeds in a set fruit. When an unfertilized spent flower falls, part of the pedicel remains attached to the plant, readily marking the former presence of a flower. Percent fruit-set is simply the proportion of fruit-bearing pedicels out of a total number of post-flowering pedicels.

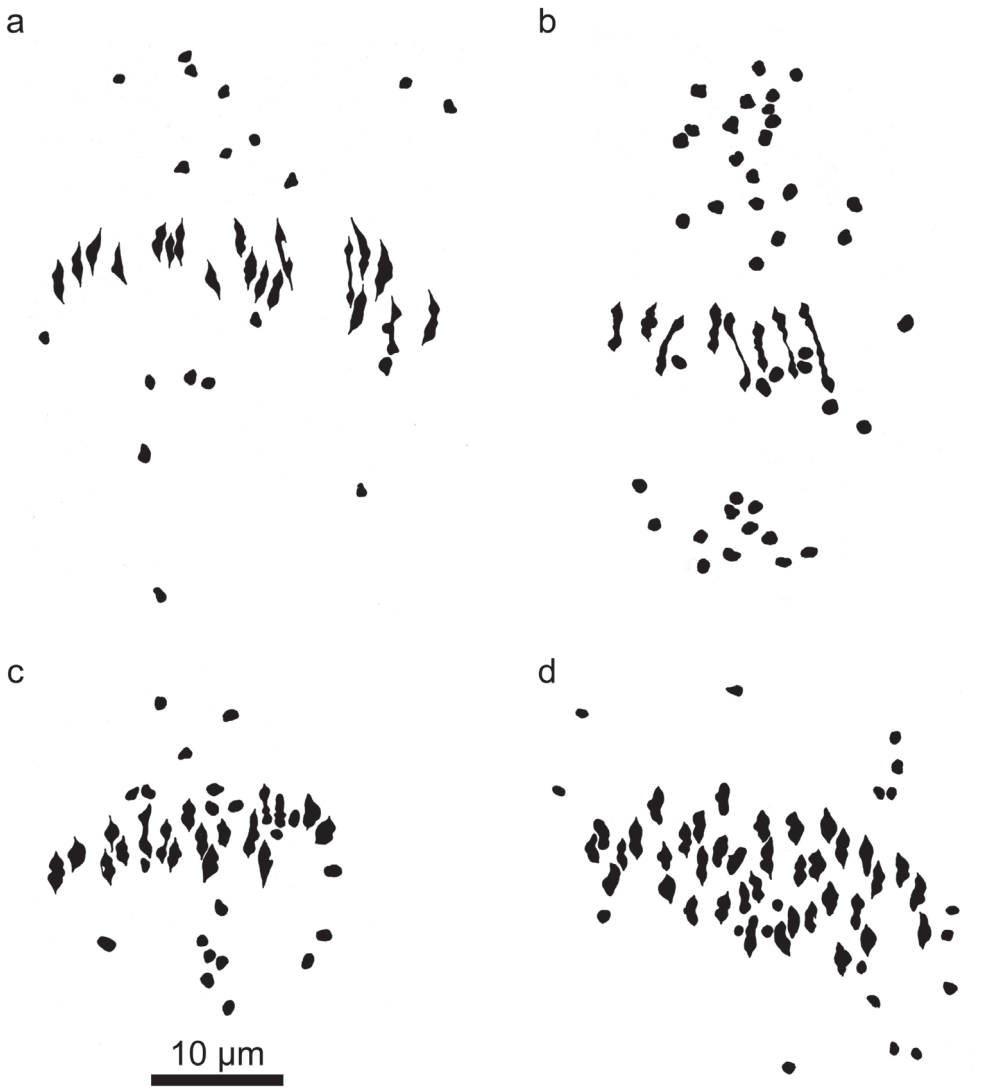

Figure 4. Camera lucida drawings of meiotic metaphase I figures in *Kosteletzkya* interspecific hybrids in which at least one parent is a polyploid. **a** *K. buettneri* × *K. borkouana* (77-160), $19_{II}+19_{I}$ **b** *K. grantii* × *K. borkouana* (77-166), $8_{II}+41_{I}$ **c** *K. grantii* × *K. begoniifolia* (81-70), $18_{II}+21_{I}$ **d** *K. begoniifolia* × *K. racemosa* (80-119), $38_{II}+19_{I}$.

Chromosome pairing

All chromosome counts and observations of chromosome pairing were made from pollen mother cells (PMCs) at meiotic metaphase I. Details of methods of collection, fixing, staining, and preservation of meiotic material can be found in Bates and Blanchard (1970) and Blanchard (2012). It was seldom possible to obtain preparations in

which all of the chromosomes were in the same plane of focus. This was not a problem for microscopic examination and interpretation, but it usually yielded less-than-satisfactory photographs (Figure 2a–c), especially of hybrids with numerous univalents that were not constrained at the metaphase plate (Fig. 2b); hence the extensive use here of camera lucida drawings. For simplicity of presentation, and because of the scale of this study, cytological outcomes are expressed in bivalent-equivalents in which the occasional quadrivalent is converted to two bivalent-equivalents, and the occasional trivalent is expressed as a single bivalent-equivalent. Unpaired chromosomes (univalents) were encountered in all crosses between different ploidy levels (e.g. Fig. 4a–d), as well as when the parents of a cross are genetically substantially divergent (e.g. Fig. 2b, 3b, 5a), and these too were counted, at least earlier in the investigation. Again, however, for the sake of clarity, they are not tabulated, but should be understood to have been present. For most hybrids at least five PMCs were examined; in more that half of the cases, more than 10 were examined.

Results

A total of 56 interspecific hybrid combinations were obtained during the course of the study (Table 3). Mean values and numbers of observations are presented for percent pollen stainability, percent fruit-set and number of chromosome bivalent-equivalents in these hybrids.

New-World interspecific crosses

All seven available New World species were involved in the crossing program (an eighth species, *K. thurberi*, was unavailable), and all interspecific crosses that were attempted were successful and comprised 17 of the 21 possible pairwise combinations among the seven species. Hybrid plants generally grew as vigorously as their parents under greenhouse conditions. Pollen stainability among them ranged from 15 to 98 percent, while fruit-set ranged from 2 to 66 percent. Low or high values in one measure did not necessarily correspond with those of the other measure. For instance, the combination *K. blanchardii* Fryxell, 1977 × *K. ramosa* had 90 percent pollen stainability but only 7 percent fruit-set.

Despite wide morphological differences among the New-World species, nearly complete chromosome pairing, as indicated by the average number of bivalents, was found in each of the 15 hybrid combinations that were examined meiotically (Table 3). Average values ranged from 18.5 to 19 bivalents out of a possible 19. As an example, Fig. 3c shows a meiotic metaphase figure from the hybrid *K. depressa* (Linnaeus, 1753) O. J. Blanchard, Fryxell et D. M. Bates, 1978 × *K. tubiflora,* whose parental species are dramatically different in habitat, morphology and floral adaptations. *Kosteletzkya depressa* is a lowland, bee-pollinated plant with a small, white-to-pink, rotate

Table 3. Experimental crosses among species of *Kosteletzkya*, showing mean values and numbers of observations (N) for pollen stainability, fruit-set and chromosome pairing in the hybrids. Hybrids are divided into New-World, African, and trans-Atlantic crosses, plus crosses with an artificial tetraploid. Species names are abbreviated using the first three letters of the specific epithet of species listed in Table 1. Names of polyploid taxa are bolded. The maximum potential number of chromosome pairs for any particular hybrid combination is also shown, in bold, in the right-most column of the table. Note that in a few cases a particular interspecific hybrid combination was made using more than one specific pair of parent plants (see, for example, bue × **sem**).

cross	parental greenhouse numbers	hybrid greenhouse number	% pollen stainability		% fruit set		bivalent equivalents		maximum potential pairing
			mean	N	mean	N	mean	N	
NEW WORLD									
bla × dep	74-9 × 74-10	75-144	**42**	7	**20**	584	**19**	16	**19**
bla × his	74-6 × 74-10	75-135	**86**	5	**27**	724	**19**	13	**19**
bla × pen	74-19 × 74-10	75-159	**15**	6	**2**	127	**18.9**	10	**19**
bla × ram	88-10 × 88-1	89-5	**90**	3	**7**	71	**19**	6	**19**
bla × tub	74-24 × 4-10	75-161	**56**	6	**25**	4	**18.7**	6	**19**
dep × pen	74-19 × 4-9	75-150	**25**	5	**14**	224	**18.5**	13	**19**
dep × his	74-9 × 74-6	75-178	**39**	5	**41**	758	**18.9**	11	**19**
dep × ram	88-9 × 88-1	89-11	**39**	3	**26**	186	**-**	-	
dep × rec	88-9 × 88-15	89-12	**51**	3	**49**	390	**19**	3	**19**
dep × tub	74-9 × 74-24	75-149	**51**	5	**29**	143	**19**	25	**19**
his × pen	74-6 × 74-15	75-127, 75-157	**79**	5	**11**	210	**18.9**	14	**19**
his × ram	88-23 × 88-1	89-7	**95**	3	**65**	147	**-**	-	
his × rec	88-23 × 88-15	89-1	**94**	3	**54**	247	**19**	8	**19**
his × tub	74-6 × 74-24	75-151	**91**	6	**49**	138	**19**	17	**19**
pen × rec	88-8 × 88-15	89-6	**78**	3	**38**	343	**18.8**	9	**19**
pen × tub	74-19 × 74-24	75-168	**73**	6	**31**	88	**18.9**	9	**19**
rec × ram	88-15 × 88-1	89-3, 89-9	**98**	6	**66**	154	**18.9**	7	**19**
AFRICA (polyploid species **bolded**)									
ado × bue	76-36 × 76-22	77-145	**0**	5	**0**	113	**3.1**	10	**19**
ado × gra	75-191 × 76-2	77-153	**1**	5	**0**	37	**2.0**	25	**19**
bue × gra	76-2 × 76-22	77-158	**0**	5	**0**	112	**9.1**	17	**19**
bue × **beg**	76-22 × 76-1	77-100, 81-26	**0**	5	**0**	252	**3.9**	25	**19**
	79-28 × 79-53	81-31	**-**	-	**0**	218	**-**	-	
bue × **bor**	76-22 × 76-40	77-160	**0**	5	**0**	21	**18.8**	12	**19**
bue × **sem**	90-27 × 90-2	91-34	**-**	-	**-**	-	**8.5**	6	**19**
	90-8 × 90-2	91-39	**-**	-	**-**	-	**4.4**	25	**19**
	90-25 × 90-2	91-10	**-**	-	**-**	-	**6.1**	15	**19**
gra × **beg**	79-41 × 79-21	81-70, 81-74	**2**	3	**0**	247	**17.9**	15	**19**
gra × **bor**	76-2 × 76-40	77-166	**0**	5	**0**	10	**7.0**	3	**19**
gra × **sem**	90-2 × 89-71	91-37	**-**	-	**-**	-	**13.1**	9	**19**
beg × bor	76-1 × 76-40	77-142	**5**	5	**0**	171	**24.6**	14	**38**
beg × rot	79-53 × 80-104	81-76	**97**	4	**63**	182	**37.0**	2	**38**
bor × sem	90-36 × 90-2	91-8	**-**	-	**-**	-	**3.5**	15	**37-38**
rot × sem	90-2 × 90-20	91-4	**-**	-	**-**	-	**11.3**	20	**37-38**
beg × rac	79-21 × 79-24	80-119	**42**	5	**0**	234	**37.1**	9	**38**

cross	parental greenhouse numbers	hybrid greenhouse number	% pollen stainability		% fruit set		bivalent equivalents		maximum potential pairing
			mean	N	mean	N	mean	N	
bor × rac	79-44 × 79-24	80-115, 80-116	**5**	5	**0**	671	**37.8**	4	**38**
	79-31 × 79-24	80-117	-	-	**0**	147	-	-	
rot × rac	80-104 × 79-24	81-73	**33**	3	**0**	141	-	-	
sem × rac	90-2 × 90-5	91-3	-	-	-	-	**6.6**	11	**37-38**
TRANS-ATLANTIC (African species listed first; polyploid species **bolded**)									
ado × dep	74-22 × 74-9	75-130, 75-156, 77-136	**7**	8	**1**	143	**1.2**	13	**19**
bue × bla	74-11 × 74-10	75-145	**26**	10	**1**	155	**19**	10	**19**
bue × dep	74-11 × 74-9	75-104	**32**	14	**2**	264	**18.9**	13	**19**
bue × his	74-11 × 74-6	75-180	**37**	5	**8**	245	**19**	10	**19**
bue × pen	74-11 × 74-19	75-148	**27**	7	**0**	129	**19**	11	**19**
bue × ram	88-18 × 88-1	89-8	**74**	3	**14**	29	**18.9**	8	**19**
bue × rec	88-6 × 88-15	89-4	**35**	3	**0**	47	-	-	
bue × tub	79-28 × 78-14	81-91	**61**	3	**4**	75	**19**	5	**19**
gra × bla	76-2 × 76-21	77-113	**0**	5	**0**	791	**11.4**	10	**19**
gra × dep	76-2 × 75-136	77-115	-	-	-	-	**7.7**	13	**19**
gra × pen	79-41 × 80-142	81-159	**0**	3	**0**	75	-	-	
beg × bla	76-1 × 76-21	77-104	**1**	4	**0**	272	**6.1**	14	**19**
bor × dep	76-40 × 75-136	77-183	**4**	5	**0**	67	**18.0**	1	**19**
bor × bla	76-40 × 76-21	77-167	-	-	-	-	**18.6**	11	**19**
bor × tub	76-40 × 76-23	77-173	**0**	5	**0**	17	**18.7**	6	**19**
sem × bla	90-2 × 90-19	91-1	-	-	-	-	**4.3**	19	**19**
sem × his	90-2 × 90-39	91-35	-	-	-	-	**7.0**	17	**19**
sem × tub	90-2 × 90-11	91-36	-	-	-	-	**10.9**	15	**19**
CROSSES WITH ARTIFICIAL TETRAPLOID (polyploid taxa **bolded**)									
art × ado	80-103 × 80-107	81-89	-		-		**19**	4	**19**
art × gra	80-103 × 79-41	81-87	-		-		**19**	5	**19**
art × beg	80-103 × 79-53	81-79	-		-		**36.9**	13	**38**
art × rot	80-103 × 80-104	81-83	-		-		**36.6**	15	**38**

corolla (petals 0.8–1 cm long), included staminal column, and a green calyx (Fig. 6B); *K. tubiflora* is an upland, apparently bird-pollinated plant with a large, yellow, tubular corolla (petals 2.5–3 cm long), exserted staminal column and a pink-to-red calyx (Fig. 6G). The two species also differ markedly in fruit and seed characteristics.

African interspecific crosses

All three possible hybrids among the three known African diploid species *K. adoensis* (A. Richard, 1847) Masters, 1868, *K. buettneri* and *K. grantii* (Masters, 1868) Garcke, 1880 were obtained, although these offspring were not as robust as the New-World

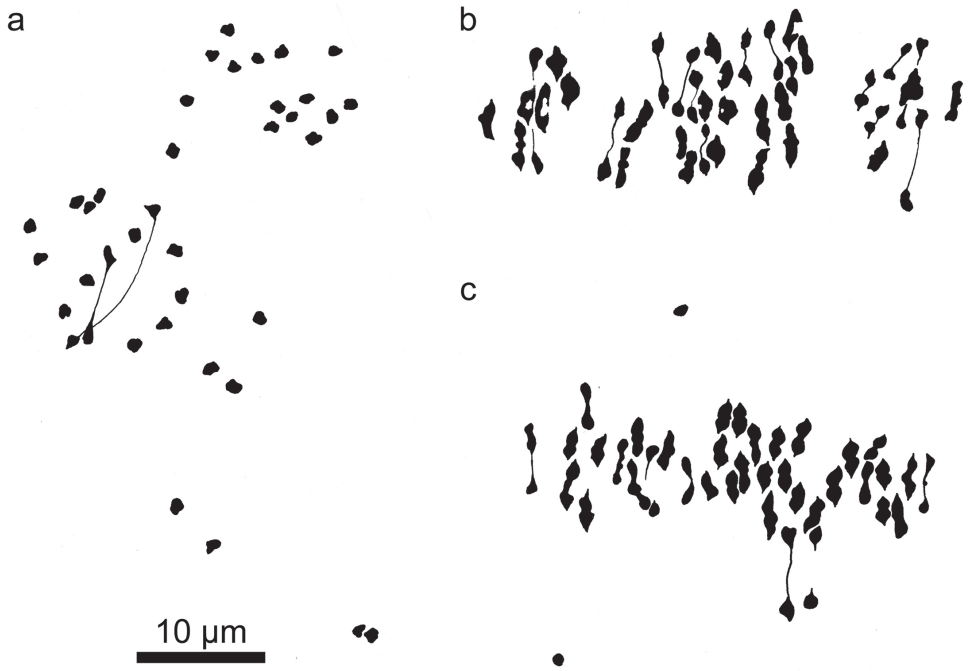

Figure 5. Camera lucida drawings of meiotic metaphase I figures in *Kosteletzkya* interspecific hybrids and an artificial tetraploid. **a** *K. adoensis* × *K. grantii* (77-153), $2_{II}+34_{I}$ **b** artificial tetraploid (82-90), 38_{II} **c** *K.* artificial tetraploid × *K. begoniifolia* (81-79), $37_{II}+2_{I}$.

hybrids. Like the New-World diploid species, the three African parent species differ considerably in habit, leaf shape, inflorescence form and details of flowers (see Figs 6H, L, O and P), fruits and seeds. However in dramatic contrast to the diploid New-World hybrids, pollen stainability in the African diploid hybrids ranged from 0 to 1 percent, fruit-set was 0 percent, and average chromosome pairing ranged from 2.0 to 9.1 out of a potential 19 bivalent-equivalents. A meiotic metaphase I of the hybrid *K. adoensis* × *K. grantii* is shown in Fig. 5a.

Hybrids between African diploids and African tetraploids were generally obtained with more difficulty, particularly in the case of the diploid *K. adoensis*, in which, to cite the most extreme example, several hundred cross-pollinations with *K. begoniifolia* (Ulbrich, 1917) Ulbrich, 1924 resulted in only a single viable seed. Nevertheless, altogether six of the possible 12 diploid-tetraploid hybrids were eventually produced. As might be expected in hybrids between ploidy levels, pollen stainability was low (0-2 percent in the four interspecific combinations that were sampled for this characteristic) and their fruit-set was likewise low (0 percent in the same four combinations). Average chromosome pairing in these six hybrids varied widely. Two approached the potential maximum of 19, forming 17.9 to 18.8 pairs (Figs 4a and 4c), while the other four ranged in average from 3.9 to 13.1 bivalent-equivalents (Figs 4b).

No diploid-hexaploid hybrids could be obtained despite numerous cross-pollinations.

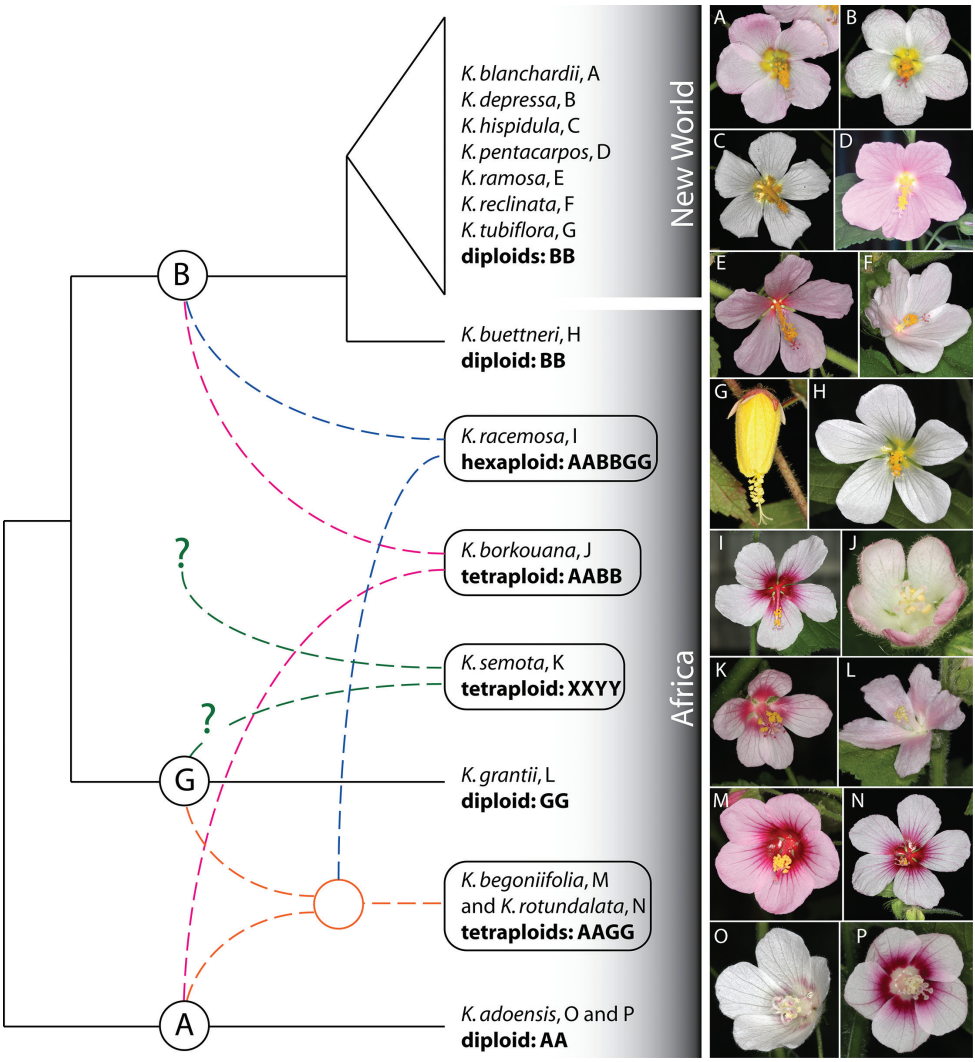

Figure 6. A representation of hypothesized phylogenetic and phytogeographic relationships among 15 species of *Kosteletzkya* based on chromosome pairing in experimental hybrids, plus photographs of the 15 species. Extant allopolyploids are shown in boxes. Distinct genomes are identified by the letters A, B, G, X and Y. Differently colored dashed lines indicate how these ancestral parents are thought to have combined to form the present-day polyploid species. One of the two alternate derivations of *K. racemosa*—that in which the B genome is carried by the diploid ancestor—is shown here. Question marks indicate uncertainties about the origins of the postulated diploid ancestors of *K. semota*. Letters after the names of taxa refer to the flower photographs to the right. The latter are not all shown to the same scale. Plants from which the flower images were made are indicated by the following greenhouse numbers (see Table 2): **A** 10-26 **B** 10-18 **C** 10-44 **D** 10-81 **E** 10-23 **F** 10-5 **G** 10-22 **H** 10-59 **I** 10-45 **J** 10-21 **K** 10-100 **L** 10-104 **M** 10-64 **N** 10-53 **O** 10-14 **P** 10-31.

The African polyploids could be crossed fairly easily among themselves, and eight of the ten possible hybrids were produced. In the five cases where pollen stainability and fruit-set were determined, stainability ranged from 5 to 97 percent, whereas fruit-set for four of the hybrids was 0 percent, while for a fifth (*K. begoniifolia* × *K. rotundalata*) it was 63 percent. Of the four tetraploid-tetraploid hybrids that were examined cytologically, one had bivalent-equivalents approaching the maximum possible 38, whereas the other three ranged from 3.5 to 24.6 bivalent-equivalents (Fig. 2b). Of the three tetraploid-hexaploid hybrids examined cytologically, two averaged over 37 pairs out of a potential maximum of 38 (Fig. 4d), while the third averaged only 6.5 bivalent-equivalents.

Trans-Atlantic interspecific crosses

Eleven of the 21 possible diploid-diploid trans-Atlantic combinations were produced. At least four failed attempts involved *K. adoensis* as one potential parent. One combination that was obtained, *K.grantii* × *K. depressa,* produced flower buds that aborted between meiosis and flowering, making pollen stainability and fruit-set data impossible to obtain. In another combination, *K. adoensis* × *K. depressa*, though the plants flowered, they were weak-stemmed and slow-growing, and produced only a feeble root system. The 10 surviving hybrids had pollen stainabilities and fruit-sets that were intermediate, on average, between those of New World diploid-diploid crosses and those of the African diploid-diploid crosses, and ranged from 0 to 74 percent pollen stainability and 0 to 14 percent fruit-set. However, depending on which of the African diploids participated, the pollen-stainability outcomes were different: in the seven African-New World crosses involving *K. buettneri*, stainability ranged from 26 to 74 percent; in the two crosses involving *K. grantii*, stainability was zero percent in both cases; and in the single cross involving *K. adoensis*, the result was seven percent pollen stainability. Outcomes of chromosome pairing observations were even more distinctly different depending on which African parent was involved. In all six combinations in which *K. buettneri* was the African parent, pairing closely approached the maximum possible 19 (see for example Fig. 3d). However when *K. grantii* or *K. adoensis* were involved, the pairing in the three hybrids that were examined cytologically ranged from 1.2 to 11.4 bivalent-equivalents (see for example Fig. 3b).

In the case of trans-Atlantic crosses between African tetraploids and New-World diploids there was again a bimodal pattern. In three of the seven hybrid combinations examined meiotically, chromosome pairing approached the maximum 19; in the other four the range was 4.3 to 10.9.

As was the case for African-African crosses, no diploid-hexaploid trans-Atlantic combinations could be obtained despite numerous crossing attempts.

Discussion

Genome differentiation and identification

In clear contrast to the nearly perfect chromosome pairing (18.5–19 bivalent-equivalents) in all of the 17 diploid New-World hybrids, the three African diploids contain chromosome sets with only low-to-modest affinity among themselves (2.0, 3.1 and 9.1 bivalent-equivalents). A consequence of this is mirrored in the negligible pollen stainability (0 to 1 percent) and zero fruit-set in hybrids among the three African species. This has prompted the designation here of three distinct genomes among the African diploids: A for the *K. adoensis* genome, B for the *K. buettneri* genome and G for the *K. grantii* genome.

Considering chromosome-pairing relationships in these genomic terms, it is apparent that only one genome is shared by all of the New World species. More interestingly, the trans-Atlantic crosses between the African diploid *K. buettneri* and six different species from the New World show a nearly perfect pairing in each, consisting on average of 18.9 to 19 bivalent-equivalents. Clearly then, the one New-World genome must be B. Indirect support for this comes from the fact that New-World species recognize only 7.7 to 11.4 chromosomes in the G genome, and only 1.2 chromosomes in the A genome—a pattern similar to crosses of these same two genomes directly with *K. buettneri* itself. These results, of course, indicate a direct connection between the African and New World parts of the genus. Simply stated, the African *K. buettneri* appears to be more closely related to the New World species than it is to its two African diploid congeners.

Genomes and polyploids

Allopolyploidy is an important mechanism for speciation in plants (Otto and Whitton 2000, Soltis et al. 2004, Wood et al. 2009), and polyploid series among related species are often found to have resulted from this process. In the Malvaceae: Malvoideae, allopolyploidy has been extensively documented in *Gossypium* (Endrizzi et al. 1985) and in *Hibiscus* sect. *Furcaria* (Wilson 1994). In *Hibiscus* sect. *Furcaria,* all of the 41 genomically studied polyploid species are allopolyploids; in *Gossypium,* all of the five known polyploid species are allopolyploids. On this basis I have hypothesized that the polyploid species in *Kosteletzkya* will prove to be allopolyploids as well.

In classic allopolyploidy, the production of an interspecific hybrid is the first step leading to a new species, yet in an examination of over 2800 herbarium specimens comprising all 17 species in the genus, no plants were found that might have been considered natural hybrids. In a way, this is not a surprise. The New-World species, though relatively easily inter-crossable, are at present largely geographically allopatric, and even where they are in geographic proximity they are kept separate elevationally (e.g. *K. depressa* and *K. tubiflora* in western Mexico) or by flowering season (e.g. *K. de-*

pressa and *K. pentacarpos* in western Cuba and southern Florida). In contrast, while the African diploids are broadly sympatric, though perhaps often ecologically separated, experiments reported here have shown that hybrids among them are more difficult to obtain and weaker—conditions likely to pertain in the wild as well. In either hemisphere the hybrids themselves would obviously be transitory, living for a few years and then likely vanishing unrecognized and leaving few if any progeny.

Nevertheless, there appears to be clear evidence of allopolyploidy in Africa. The experimental diploid-tetraploid hybrid between *K. grantii* (2x) and *K. begoniifolia* (4x) averaged 17.9 bivalent-equivalents out of a possible 19, and likewise the diploid-tetraploid hybrid between *K. buettneri* (2x) and *K. borkouana* (4x) averaged 18.8 out of 19. The reverse crosses (*K. buettneri* × *K. begoniifolia* and *K. grantii* × *K. borkouana*) yielded averages of 3.9 and 7.0 respectively. This suggests that the G genome but not the B genome is present in the tetraploid *K. begoniifolia*, and the B genome but not the G genome is present in the tetraploid *K. borkouana*. Neither of these two diploids was combined in this study into a hybrid with the tetraploid *K. rotundalata* but this species shows nearly complete chromosome homology with *K. begoniifolia*—an average of 37.0 pairs out of a potential 38—suggesting a close relationship between the two, and indirectly indicating that the G genome is also present in *K. rotundalata*. Finally, the tetraploid *K. semota* was combined with both the B-bearing *K. borkouana* and the G-bearing *K. rotundalata*, producing on average only 3.5 and 11.3 pairs respectively, from which it can be reasonably concluded that *K. semota* contains neither B nor G genomes. This is further suggested by crosses of *K. semota* with the African diploids *K. buettneri* and *K. grantii* as well as with three New-World B-genome diploids, in which all five results ranged between 4.3 and 13.1 bivalent-equivalents.

When the tetraploids *K. begoniifolia* and *K. borkouana* were crossed with one another, the resulting hybrids averaged 24.6 bivalent-equivalents out of a possible 36, which indicates that they share a genome. That genome cannot be either G or B since it is shown above that neither is shared by the two tetraploids. This shared, unknown *K. borkouana-K. begoniifolia-K. rotundalata* genome cannot be present in *K. semota* because many fewer that a full set of 19 chromosomes were detected in *K. semota* by these other tetraploids.

The artificial tetraploid

Although one might invoke some undiscovered or now-extinct genome as the postulated shared genome, the most obvious suggestion is that it is the extant genome A. It was therefore particularly frustrating that hundreds of cross-pollinations between the A-bearing *K. adoensis* and the tetraploids *K. begoniifolia* and *K. borkouana* produced only a single viable seed—from the first of these two combinations—and that seed yielded a severely stunted, deformed, non-reproductive plant. This made it impossible to introduce an A genome into either of the two tetraploids to the extent that it could reach meiosis and seek out a possible genomic match. On the other hand, the

putative hybrids that would have had to form in nature as a first step in the allopolyploid production of the postulated AAGG and AABB tetraploids are, respectively, the combinations AG (*K. adoensis* × *K. grantii*), and AB (*K. adoensis* × *K. buettneri*). Both of these hybrids were indeed produced experimentally. The first had 1 percent pollen stainability, 0 percent fruit-set and produced an average of 2.0 bivalent-equivalents out of a potential 19; the second had 0 percent pollen stainability, 0 percent fruit-set, and an average of 3.1 bivalent equivalents. Fig. 5a illustrates a meiotic metaphase I of the AG hybrid.

Remarkably, one day I found in the greenhouse a normal-appearing fruit on a branch of the otherwise profoundly sterile diploid hybrid AG plant. It contained three well-formed seeds, all of which subsequently germinated and produced vigorous plants which themselves yielded abundant fruit when selfed. This restoration of fertility strongly suggested that a spontaneous doubling of chromosomes had occurred in at least a small part of the hybrid plant, thereby creating identical pairs of chromosome sets and permitting full synapsis in meiotic prophase I, with the result that meiosis and gamete formation were able to proceed to normal completion. Examination of meiotic metaphase in one of these plants indeed showed 38 pairs of chromosomes (Fig. 5b). The conclusion: a combination of experimental and accidental events had resulted in a new, fully fertile tetraploid. A test of this would be a cross between this artificial tetraploid and one of its wild putative counterparts, *K. begoniifolia* or *K. rotundalata*. Both of these test hybrids were obtained, and they showed averages of 36.9 and 36.6 bivalent-equivalents respectively out of a potential 38. Fig. 5c shows a meiotic figure illustrating the combination *K. begoniifolia* × *K.* artificial tetraploid.

This settled two important matters: 1) that the artificial tetraploid corresponded genomically to these wild tetraploids, and 2) that the identity of the elusive shared genome was indeed A. The latter was also separately verified by subsequent backcrossing of the artificial tetraploid to each of its two diploid parents. These crosses with *K. adoensis* and with *K. grantii* each yielded an average pairing of 19 out of 19 potential pairs. Interestingly, *K. adoensis*, which had been so intractable in attempts at crossing it with the two wild tetraploids, crossed fairly readily with their home-made counterpart, and the offspring grew and flowered well.

In summary, these results suggest that the genomic makeups of the tetraploids *K. begoniifolia, K. rotundalata* and *K. borkouana* are respectively AAGG, AAGG and AABB.

Since it is generally observed that most allopolyploids arise via unreduced gametes (de Wet 1971, Ramsey and Schemske 1998), and since the actual initiation of an allopolyploid event is rarely witnessed, it is noteworthy that the spontaneous polyploidization reported here was apparently due not to unreduced gametes but to somatic doubling. Part of the plant—perhaps only a single flower—must have arisen from a chromosome doubling in a somatic apical initial or an early derivative. A contrary interpretation would require the unlikely independent production, within only a single flower and no other flowers on the plant, of a minimum of six unreduced gametes—three eggs and three sperm—which then would have to meet by chance and go on to result in a single capsule bearing three fertile seeds out of a potential five.

The hexaploid *Kosteletzkya racemosa* Hauman, 1961

Despite numerous attempts, no hybrids could be produced between the single African hexaploid *K. racemosa* and any of the diploids, either African or New-World, however the hexaploid did cross with all four tetraploids. With *K. begoniifolia* and *K. borkouana* it averaged 37.1 and 37.8 chromosome pairs respectively out of a possible 38 (Figure 4d), whereas with *K. semota* it showed 6.6 pairs out of a possible 38. (Meiotic material from the hybrid *K. rotundalata* × *K. racemosa* was not obtained.) Now that genomic makeups are known for the tetraploids *K. borkouana* and *K. begoniifolia* it can be stated with reasonable certainty that the genomic constitution of the hexaploid is AABBGG. This is because both AAGG (*K. begoniifolia*) and AABB (*K. borkouana*) tetraploids were shown separately to find nearly perfect correspondence with two sets of chromosomes in the hexaploid.

The hexaploid could theoretically have arisen in nature in one of two ways. A triploid ABG hybrid could have formed between an AABB tetraploid and a GG diploid, or between an AAGG tetraploid and a BB diploid, in both cases followed by chromosome doubling. No persuasive evidence at present strongly favors either scenario as being more likely, but the *K. buettneri*-like narrower leaves and depressed fruit, plus the *K. begoniifolia*-like dark petal bases and larger seeds that together characterize the hexaploid, hint that the combination AAGG × BB might be the better candidate. The matter is complicated by the fact that while both of the relevant diploids occur in the geographical vicinity of the two known occurrences of *K. racemosa* in southern Congo-Kinshasa and northwestern Zambia, none of the tetraploids are currently known to do so.

The preceding discussion is not meant to imply that *K. adoensis, K. buettneri* and *K. grantii* are the direct, immediate sources of the genomes A, B and G that are found in the polyploids, but rather that the lineages that gave rise to these three modern diploids also contributed their genomes relatively recently to the polyploids. The two interspecific hybrids that are the diploid counterparts, AB and AG, of the natural tetraploids whose genomic makeups are known, i.e. *K. borkouana* (AABB) and *K. begoniifolia/rotundalata* (AAGG), are only somewhat similar morphologically, not identical, to these tetraploids. Likewise the artificial tetraploid that is the full genomic counterpart of the wild species *K. begoniifolia* is similar to the latter, but distinguishable from it. Finally, both the triploid combination *K. begoniifolia-K. buettneri* (ABG), and the triploid combination *K. borkouana-K. grantii* (also ABG), look similar, but not identical, to the hexaploid *K. racemosa* (AABBGG).

Kosteletzkya semota

Kosteletzkya semota, the third genomically distinctive tetraploid (recall that *K. rotundalata* and *K. begoniifolia* are genomically alike), appears to show little affinity with any of the three known diploid genomes. In effect the hexaploid *K. racemosa* offered all three known genomes to *K. semota* in a cross, but the 37-38 chromosomes of the latter could only recognize, on average, 6.6 *K. racemosa* chromosomes—the equivalent

of about one-third of a genome. There were similar outcomes when *semota* participated in crosses with other tetraploids discussed above, yielding bivalent-equivalents ranging from 3.5 to 11.3, indicating at best a low-to-modest level of chromosomal homology with any of the other known genomes. Interestingly however, *K. semota* was shown to share over two-thirds of a genome (13.1 pairs) in a cross directly with the diploid *K. grantii*. These varied results leave uncertainties about the evolutionary position *K. semota*, so it seems best to give it a provisional designation of XXYY, which recognizes the species as distinctive and assumes an allopolyploid origin. At least one of its diploid progenitors, and its constituent genome, remains undiscovered or, more likely, extinct.

Bivalent-equivalents

The decision to convert trivalents and quadrivalents to bivalent-equivalents (see Materials and methods) was intended to make Table 3 easier to read and interpret, but it is worth considering whether this conversion might have led to bias. In polyploid hybrids, multivalent associations sometimes indicate pairing within genomes (autosyndesis), which in turn suggests autopolyploidy—an interpretation that conflicts with the hypothesis of allopolyploidy that I have posited here. In the present case however, such an explanation is highly unlikely. Of the 618 meiotic cells examined, only nine had a single trivalent and only 18 others had a single quadrivalent. Moreover, 24 of these 27 multivalents occurred in diploid-diploid hybrids, and therefore could not be attributed, by definition, to autosyndesis. The three exceptions, involving one trivalent and two quadrivalents, were all found in hybrids in which the artificial tetraploid was one parent, and since I have shown here that this plant was derived from an interspecific diploid-diploid hybrid, its multivalents cannot be interpreted as indicating autopolyploidy.

A hypothesized evolutionary (geographic and genomic) history of *Kosteletzkya*

Figure 6 depicts a reconstruction of the postulated genomic-phytogeographic history of the genus *Kosteletzkya* based on the cytogenetic evidence presented here. It assumes that the degree of chromosome pairing in the experimental hybrids can be used as a rough relative measure of the degree of evolutionary divergence of the parents of a cross. In support of this assumption, I note that in the well-studied malvaceous genus *Gossypium*, the African genomes A, B and E show pairing relationships among themselves (data from Konan et al. 2009) that are similar to those among the A, B, and G genomes of *Kosteletzkya*, and in the case of *Gossypium* the extent of the evolutionary divergence suggested by the degree of chromosome pairing is supported by both morphological evidence (Fryxell 1971) and molecular evidence (Cronn and Wendel 2004).

The reconstruction shown here indicates the reticulate nature of the evolution of *Kosteletzkya* in Africa, and also emphasizes that all of the early events in the history of

the genus took place on the African continent. The lineage giving rise eventually to the B and G genomes is shown as separating from the A-genome lineage early in the evolution of the genus. This B-G branch itself branched in a more recent step, leading eventually to the extant African diploids *K. buettneri* and *K. grantii* respectively, while the A genome eventually gave rise to the extant diploid *K. adoensis*. The rest of the African diversification occurred at the polyploid level, and initially involved two separate interspecific hybridizations, each of which involved an A-genome plant—one in combination with a B-genome plant and the other with a G-genome plant. Following a doubling of the chromosome complements in these two hybrids, and subsequent evolution at the tetraploid level, the two resulted in three extant species: *K. borkouana* with genomic makeup AABB, and *K. begoniifolia* and *K. rotundalata*, each with AAGG. A cross between one of these tetraploids and a diploid bearing the third genome, followed by doubling, produced the hexaploid *K. racemosa* having a genomic makeup of AABBGG. Of the two possible hybrid combinations that might have led to this hexaploid, I have illustrated the one in which the tetraploid partner is from the *K. begoniifolia-K. rotundalata* (AAGG) lineage, since this alternative seems better supported by morphological evidence. The two postulated early-diverging genomes that led to the formation of the tetraploid *K. semota* have been designated here as XXYY, but in the depiction in Fig. 6, one of its genomes is tentatively shown as having its origin in the *K. grantii* lineage, since the cross *K. semota* × *K. grantii* yielded 13.1 chromosome pairs, the equivalent of more than 2/3 of a genome.

The atypically high level of fruit-set—63 percent—in the African tetraploid-tetraploid cross *K. begoniifolia* × *K. rotundalata* contrasts dramatically with the zero percent seen in all twelve of the other African hybrids for which there are fruit-set data (Table 3). This and the pairing evidence imply that the two parents diverged relatively recently from a common tetraploid ancestor, and this, too, is suggested in Fig. 6.

Finally, the history of *Kosteletzkya* in the New World was set into motion by a relatively recent dispersal to the New World of a B-genome-bearing *K. buettneri* ancestor, followed by a rapid radiation to yield the seven known diploids in that hemisphere. A similar pattern, in which one among several African genomes is also found in the New-World, can be seen in *Gossypium* (Endrizzi et al. 1985, Wendel and Cronn 2003) and in *Hibiscus* sect. *Furcaria* (see Menzel et al. 1983), and in both cases trans-Atlantic dispersals to the New World have been invoked**.**

In reporting on chromosome numbers in *Kosteletzkya*, I used the numbers evidence to suggest that Africa was the birthplace of the genus (Blanchard 2012). The data newly presented here add strength to this contention. The genomic and ploidy profiles of the African half of the genus are so strikingly more deep and complex than in the New World half as to lead almost inevitably to the view that, despite similar levels of morphological diversity, the New World taxa are of a much more recent origin.

Within the diversity of the New-World *Kosteletzkya* species there is little likelihood that any as-yet-undiscovered polyploids exist. Any interspecific New-World hybrid that formed would contain two B genomes, and in the event of a chromosome doubling, there would be four closely similar sets of chromosomes entering prophase I of meiosis.

The result, barring strong preferential pairing, would be multivalent associations, confused and uneven segregation at anaphase, and consequently much reduced fertility**.**

An extension of this idea may explain why genome A, rather than one of the other two African genomes, is the one that is shared among the tetraploids *K. begoniifolia, K. borkouana* and *K. rotundalata*. While there is some disagreement about whether or not allopolyploidy occurs more commonly in hybrids between parents with a greater genetic distance between them (Chapman and Burke 2007, Buggs et al. 2008, Paun et al. 2009, Buggs et al. 2011), the situation in *Kosteletzkya* suggests that distance may count. Of the three pairwise combinations among the African diploids, only the two combinations AB and AG, have truly low levels of chromosome pairing—averaging only about 2 or 3 bivalents out of a potential 19. It is these two combinations that have been shown here to have given rise to the tetraploids *K. borkouana* and *K. begoniifolia/K. rotundalata* respectively. The third combination, BG, averages 9.1 bivalents—the equivalent of nearly half a genome. If a hybrid of the latter were to have formed and experienced a doubling of its chromosomes it would be much more likely than the other two to suffer serious meiotic problems. There is no present-day evidence of such a polyploid, and its existence would not be expected.

Long-distance dispersal

The suggestion that a *Kosteletzkya* carrying a B genome made its pre-Columbian way across the Atlantic calls for an evaluation of the dispersal capabilities of the group. Stephens (1966) discussed the matter for *Gossypium*, which presents a similar problem of an amphi-Atlantic distribution of its A genome. Hochreutiner, in his revision of the genus *Hibiscus* (1900), considered the winged fruits of species of *Hibiscus* sect. *Pterocarpus* Garcke, 1849 and found no dispersal function for the wings since the capsules of *Hibiscus* dehisce in place to release their seeds. Instead he suggested that the wings mimicked those of the fruits of an East-African *Pavonia* in which, however, the wing-bearing mericarps actually individually enclose the seeds and could therefore presumably act as windborne disseminules. He later elaborated further on winged fruits in the Malvoideae (1913). Mattei (1917) rejected Hochreutiner's interpretation and also extended the discussion to *Kosteletzkya*, saying that in both *Hibiscus* sect. *Pterocarpus* and in *Kosteletzkya* the capsule-valves separate from the fruiting axis, so that it was possible that incompletely laterally disarticulated capsule-valve pairs could become windborne and carry seeds with them. Hochreutiner later (1924) conceded that his earlier comments about mimicry had been weak, but he maintained that in his experience all of the capsule-valves in these plants separated at the same time from the axis, not in groups. He proposed instead that the wings were organs for dehiscence of the capsule, not for seed dissemination, and cited other not really comparable examples from elsewhere in the Malvoideae.

My experience with *Kosteletzkya* indicates that both Mattei and Hochreutiner were correct. Adjacent capsule-valves in *Kosteletzkya* often do cohere in pairs or threes and

separate together from the fruiting axis, carrying one or two seeds with them (Blanchard in Verdcourt and Mwachala 2009). This can easily be observed when one harvests seeds by manually stripping a fruiting branch of its mature capsules. Many capsules fall completely apart with this rough handling, but some do not. Equally importantly, I found that it was not uncommon, when working in the greenhouse or collecting the plants in the wild, to discover that single or coherent capsule-valves had attached to clothing. The fruits of nearly all *Kosteletzkya* have bristly, sometimes hooked hairs either covering the whole surface or confined to the valve margins. These certainly are responsible for the adhesion, and I have no doubt that they may cling to fur and feathers as well, and aid in the dispersal of the seeds. To the extent that the wings help the bristles to project from the general fruit surface and therefore to be better exposed to passers-by, the wings aid in dispersal, but hardly in the form of windborne "flight."

Kosteletzkya pentacarpos has been shown (as *K. virginica* [Linnaeus, 1753] A. Gray, 1849) to have an air space within the seed that permits it to float (Poljakoff-Mayber et al. 1992), and the same species has been credited with considerable salt tolerance and a seed coat that remains impermeable to water for some time (Poljakoff-Mayber et al. 1994). These features may aid in salt-marsh-to-salt-marsh dispersal as they apparently do in *Hibiscus moscheutos* Linnaeus, 1753, with which *K. pentacarpos* often shares habitat (Kudoh et al. 2006), and it is possible that the same characteristics could enhance the prospects of a trans-Atlantic crossing.

Dispersal over considerable distances appears to have been accomplished by several *Kosteletzkya* species. Known contemporary distributions suggest that *K. adoensis* has jumped from the African mainland to Madagascar, which is a minimum of 800 miles from the nearest known mainland population. It also appears to have dispersed westward from its main center in East Africa to the mountains of Cameroon (1100 miles) and the mountains of Sierra Leone (a further 1350 miles), with no known occurrences of the species—and no montane habitats—in the intervening areas. *K. begoniifolia* has made a similar jump from montane East Africa to Cameroon (1050 miles), and *K. borkouana* has dispersed from eastern Democratic Republic of the Congo and East Africa 1300 miles across a considerable expanse of the Sahara to the Borkou region of northern Chad (Blanchard 2013). In the New World, *K. depressa* has spread to the Cayman Islands and throughout the Greater Antilles, presumably from a mainland source (see Howard 1973), and *K. pentacarpos* has spread from the United States to Cuba and Bermuda. Moreover, if the latter turns out not to have been transported to Eurasia by human agency, it must have made the trans-Atlantic trip by long-distance dispersal, as did the carrier of the ancestral B-genome, but in this case dispersing from west to east.

It is worth noting, however, that some of these dispersal feats may have been aided in Africa by paleoclimatic cycles that provided geographically more benign intervening conditions (Quézel 1978, de Menocal 2011). In the case of *K. borkouana*, for example, the Sahara Desert was apparently largely vegetated at times during the interval from 15000 to 5000 years ago (de Menocal et al. 2000), and may have afforded the plant an opportunity to disperse by much shorter hops to northern Chad from East Africa. In the case of the Caribbean-island immigrants, on the other hand, the time

frame in which these plants could have dispersed without having undergone appreciable subsequent divergence would have been too recent to attribute their dispersal to the narrowed or bridged ocean gaps known to have occurred earlier in the Cenozoic, so some sort of island-hopping seems to be the only plausible explanation for the island distributions of *K. depressa* and *K. pentacarpos* (see Pindell 1994).

Two *Kosteletzkya* species were unavailable for inclusion in the hybridization experiments reported here: *K. thurberi* from northwestern Mexico and the rare *K. batacensis*, endemic to the Philippine island of Luzon. It can be reasonably predicted that *K. thurberi* is a diploid of genomic makeup BB like all other New-World *Kosteletzkya* species. *Kosteletzkya batacensis*, on the other hand, remains a complete mystery. On the basis of general morphology—particularly of the fruits—the plant seems to belong in *Kosteletzkya*, although it is unique in the genus in being an annual. However its restricted range and its remote geographical location in relation to the rest of the genus would make any further speculation on its relationships almost reckless. There was an active maritime trade between Manila and Mexico for centuries, but suggestions of a Mexican origin for the plant (Merrill 1912, 1918, Borssum-Waalkes 1966), although appealing, are not supported by what is known of the several extant Mexican taxa (Blanchard 2008).

Conclusions

In the two centers of diversity of *Kosteletzkya*, Africa and the northern Neotropics, the constituent species occur in approximately equal numbers and display similar ranges of morphological diversity. The results of the present study suggest that this apparent symmetry hides profound underlying differences in the evolutionary histories of the two groups. Pairing relationships in interspecific hybrids appear to show that *Kosteletzkya* in Africa underwent an early diversification at the diploid level, followed by a rich and complex history of allopolyploidy. In dramatic contrast, *Kosteletzkya* made a late appearance in the New World, where it underwent a rapid diploid-level diversification. These observations, especially if also borne out by a molecular study currently under way, lend strong support to my earlier contention, based on chromosome-number differences, that Africa is the birthplace of the genus. This scenario necessitates a long-distance dispersal, and the fruit and seed adaptations in *Kosteletzkya*, as well as known within-hemisphere dispersals, suggest such a capability. These observations also lend further weight to similar dispersals proposed by other workers to explain distributions in other malvoid genera, including the precursors of the cultivated cottons.

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