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RESEARCH ARTICLE

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Molecular characterization of constitutive heterochromatin in three species of *Trypoxylon* (Hymenoptera, Crabronidae, Trypoxylini) by CMA_3/DAPI staining

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Abstract

Previous cytogenetic analyses in *Trypoxylon* Latreille, 1796 have been basically restricted to C-banding. In the present study, base-specific CMA_3 and DAPI fluorochrome staining were used to characterize the constitutive heterochromatin in three *Trypoxylon* species. The heterochromatin was GC-rich in all the species studied; however, in *Trypoxylon nitidum* F. Smith, 1856 the molecular composition of the heterochromatin was different among chromosome pairs. Conversely, the euchromatin was AT-rich in the three species. These results suggest high conservatism in the euchromatic regions as opposed to the heterochromatic regions that have a high rate of changes. In this study, we report the karyotype of *Trypoxylon rugifrons* F. Smith, 1873 which has the lowest chromosome number in the genus and other characteristics of the likely ancestral *Trypoxylon* karyotype.

Keywords

Comparative cytogenetics, heterochromatin, CMA3/DAPI

Introduction

The Hymenoptera (Bees, wasps and ants) have received a remarkable amount of attention due to their amazing diversity of species, life histories, social behaviors, and key role in very diverse ecosystems. Their importance arises from their role as pollinators

and biocontrol agents, their damage to agriculture and forestry, and their use as model organisms for the study of genetics and evolution (Ronquist 1999, Savard et al. 2006, Goodisman et al. 2008).

Trypoxylon Latreille, 1796 is a genus of solitary mud-dauber spider-hunting wasps that construct tubular nests entirely of mud or in preexisting tubular cavities. The genus comprises 660 species divided among two subgenera, *Trypoxylon* s.str. with a cosmopolitan distribution and *Trypargilum* Richards, 1934 restricted to the Western Hemisphere (Coville 1982, Hanson and Menke 1995).

Chromosome number and other karyological features are good sources of evidence for the systematics and species level taxonomy of several Hymenoptera groups. A marked chromosomal variability, even within species, has been reported for several aculeate Hymenoptera (Hoshiba et al. 1989). Developments in cytogenetic methods have opened the possibility of examining variation in chromosome number and structure in natural populations, therefore improving and expanding our knowledge of wasp karyology (Hoshiba et al. 1989, Gokhman and Quicke 1995, Gokhman 2010). However, cytogenetics has rarely been applied to the study of *Trypoxylon* and, so far, only 13 of the named 660 species have a known chromosome number.

In *Trypoxylon*, chromosome numbers range from 2n=18 to 2n=34 (Hoshiba and Imai 1993, Gomes et al. 1995, 1997) and two occurrences of intraspecific karyotype variation have been detected. In *Trypoxylon albitarse* Fabricius, 1804, karyotype variation was due to the presence of B chromosomes (Araújo et al. 2000). Scher and Pompolo (2003) also found remarkable karyotype differences (n=12 to 15 and 2n =25 to 28 and 2n=30) in a population of *Trypoxylon nitidum* F. Smith, 1856 in southeastern Brazil. Heterochromatin has been shown to be highly variable in the hymenopterans and has been specially meaningful in the evolution of *Trypoxylon* karyotypes due to its high content in some species (Gomes et al. 1995, 1997, Scher and Pompolo 2003).

Previous cytogenetic analyses in *Trypoxylon* have been focused on C-banding, which by itself may not be sufficiently informative for a reliable heterochromatin description and comparative analysis of different species. Therefore, other techniques for molecular characterization can be very useful for this purpose. To improve qualitatively the data available so far, we applied in combination C-banding and base-specific fluorochrome staining to the chromosomes of three *Trypoxylon* species from the Atlantic rainforest in southern Bahia, in the Brazilian Northeast.

Material and methods

Larvae of *Trypoxylon* (*Trypargilum*) *nitidum* (eight ♀ and three ♂), *Trypoxylon* (*Trypargilum*) *lactitarse* Saussure, 1867 (three ♀ and three ♂) and *Trypoxylon* (*Trypoxylon*) *rugifrons* F. Smith, 1873 (three ♀ and one ♂) were collected in the field directly in the wasp nests for cytogenetic analyses.

Specimens of *T. nitidum* and *T. lactitarse* were captured using trap-nests made of bamboo shoots sectioned below each node with 1 cm diameter or tubes of cardboard

with one end closed with the same material and 0.7 cm diameter. The trap-nests were set up in Camacan (15°25'S, 39°29'W) and Ilhéus (14°47'S, 39°12'W), in the state of Bahia and were inspected twice a week. The traps containing complete nests were closed and taken to the Laboratório de Citogenética at the Universidade Estadual de Santa Cruz for the collections of specimens in the prepupal stage. Specimens of *T. rugifrons* were captured from naturally occurring nests in Ilhéus.

At least two specimens per nest were kept at 28°C in a biochemical oxygen demand (BOD) incubator and daily monitored until adult emergence, at which time they were identified. Voucher specimens were deposited at the Entomological Collection at the Universidade Estadual de Santa Cruz.

Slides for cytogenetic analysis were prepared from cerebral ganglia of prepupae following Imai et al. (1988a). The slides containing metaphases were submitted to conventional Giemsa staining and C-banding (Sumner 1972, Pompolo and Takahashi 1990) to determine patterns of constitutive heterochromatin distribution. CMA_3/DAPI double staining followed Schweizer (1980) with minor modifications by Guerra and Souza (2002).

At least 10 metaphases per specimen were observed using an epifluorescence microscope DMRA2 (Leica) and images were captured using the IM50 Leica software (Leica Microsystems Imaging Solutions Ltd, Cambridge, UK). Giemsa-stained chromosomes were photographed using an Olympus BX51 microscope equipped with an Olympus C-7070 digital camera. Digital images and figure mounting were prepared using Adobe Photoshop CS3 Extended version 10.0.

Chromosomes were described according to Imai's terminology (Imai 1991) with acrocentric chromosome (A); pseudoacrocentric chromosome (A^M); pseudoacrocentric chromosome with a heterochromatic block in the proximal region of the centromere of the euchromatic arm (A^{MC}); metacentric heterochromatic chromosome (M^h); metacentric chromosome with an interstitial heterochromatic block in one of the arms (M^C); metacentric chromosome with an interstitial heterochromatic block (M^{CC}) (see the original reference for more details).

Results

Specimens of *T. nitidum* from Camacan had 2n=30 and n=15 chromosomes and the diploid karyotype formula was $2K=2M^{CC}+10A^M+18A$ (Figs 1A and 1B). Fluorochrome staining (Fig. 1B) revealed that pairs 1 to 5 (A^M chromosomes) showed predominantly compacted heterochromatin (equally CMA_3/DAPI stained) with balanced GC and AT composition over the long arm, except for the 1st pair, which showed a terminal euchromatic CMA_3^+/$DAPI^-$ (GC-rich) block. The heterochromatin of the acrocentric (6th to 14th pairs) and metacentric (15th pair) chromosomes showed a CMA_3^+/$DAPI^-$ pattern (Fig. 1B) whereas the remaining euchromatin in all chromosomes was CMA_3^-/$DAPI^+$ (AT-rich). Karyotypes also showed size heteromorphism on the 1st and 5th pairs (Fig. 1B).

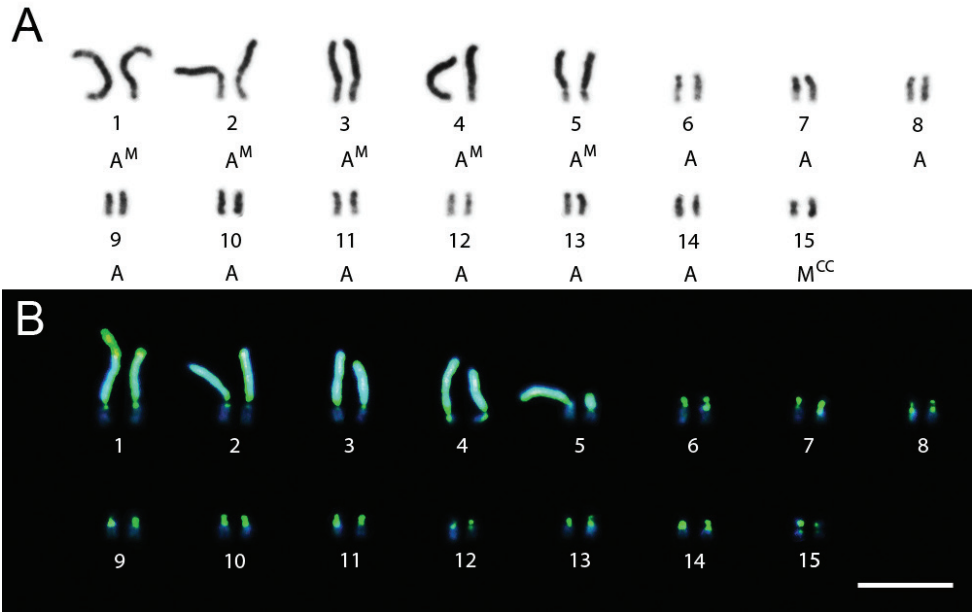

Figure 1A–B. *Trypoxylon* (*Trypargilum*) *nitidum*. **A** Female karyotype (2n=30), standard staining **B** CMA_3/DAPI staining of female karyotype (2n=30). Bar=10μm.

Specimens of *T. nitidum* collected in Ilhéus showed chromosome numbers 2n=29 and n=15 or n=13. The fluorochrome staining pattern also differed in these karyotypes. The 4th pair showed an interstitial CMA_3^+/$DAPI^-$ block near the centromeric region of one of the arms, chromosome pair 15 (Fig. 2B) had the long arm with a CMA_3^+/$DAPI^-$ pattern and chromosome pairs 11 and 13 (Fig. 2A) showed short arms with a CMA_3^+/$DAPI^-$ pattern and long arms with a CMA_3^-/$DAPI^+$ pattern.

Trypoxylon lactitarse had 2n=30 and n=15 chromosomes and the karyotype formula $2K = 2M^{CC} + 28A^{MC}$ (Figs 3A and 3B). The 4th pair was heteromorphic with variation in the length of the long arm (Fig. 3B). The heterochromatin in all chromosomes showed a CMA_3^+/$DAPI^-$ pattern, whereas the euchromatin showed a CMA_3^-/$DAPI^+$ pattern (Fig. 3B).

Trypoxylon rugifrons had 2n=16 and n=8 chromosomes and the observed karyotype formula was $2K=12M^{CC}+4A^{MC}$ (Figs 4A and 4B). The heterochromatin in all chromosomes was CMA_3^+/$DAPI^-$ and the euchromatin showed a CMA_3^-/$DAPI^+$ pattern (Fig. 4C).

Discussion

A large variation in chromosome number has been reported for several Hymenoptera groups (Imai et al. 1977, Crosland and Crozier 1986, Pompolo and Takahashi 1987, 1990, Imai and Taylor 1989, Costa et al. 1993, Gokhman 2010). Specimens of *T.*

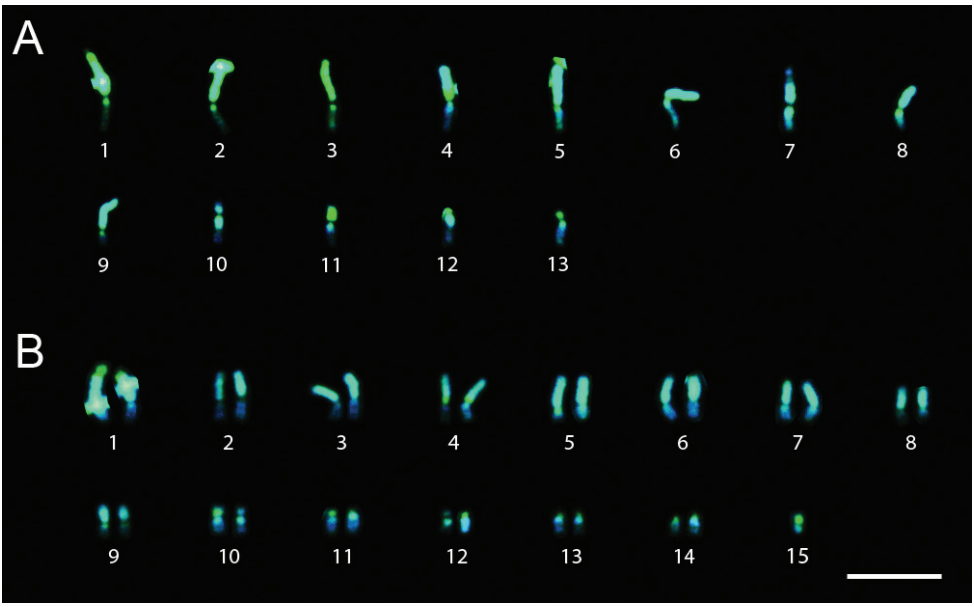

Figure 2A–B. *Trypoxylon* (*Trypargilum*) *nitidum.* **A** Male karyotype (n=13) with CMA_3/DAPI banding **B** CMA_3/DAPI staining of female karyotype (2n=29). Bar=10μm.

nitidum and *T. lactitarse* showed a chromosome number within the range observed in this subgenus, whereas *T. rugifrons* specimens showed the lowest chromosome number reported in the genus so far. The karyotypes and C-banding in *T. lactitarse* and *T. nitidum* from Camacan were similar to those found by Gomes et al. (1997) and Scher and Pompolo (2003) in the Brazilian Southeast region, revealing karyotype stability within these species along this range of the geographic distribution.

Regarding chromosome morphology, *T. rugifrons* showed predominantly metacentric and submetacentric chromosomes (pairs 1 to 6) and heterochromatin concentrated in the pericentromeric regions. These chromosomes are larger than those of the pairs 7 and 8. Gomes et al. (1997) proposed that the ancestral karyotype of *Trypoxylon* would probably have had n = 7 or 8 chromosomes and that an increase in chromosome number due to centric fissions led to the higher chromosome number registered thus far in the group. Their assumption was based on Imai et al.´s (1986, 1988a,b, 1994) minimum interaction hypothesis for karyotype evolution, which proposes that chromosome numbers tend to increase by centric fission and this process could be evolutionarily favored by the reduction of physical interaction between non-homologous chromosomes, therefore minimizing the genetic risks of deleterious translocations during meiosis.

The predominance of highly heterochromatic pseudoacrocentric chromosomes in most species within the genus *Trypoxylon* and the reduced chromosome number and chromosome morphology showed by *T. rugifrons* lend support to Gomes et al.´s (1997) hypothesis. *T. rugifrons* with n = 8 could show features similar to those of the putative ancestral karyotype.

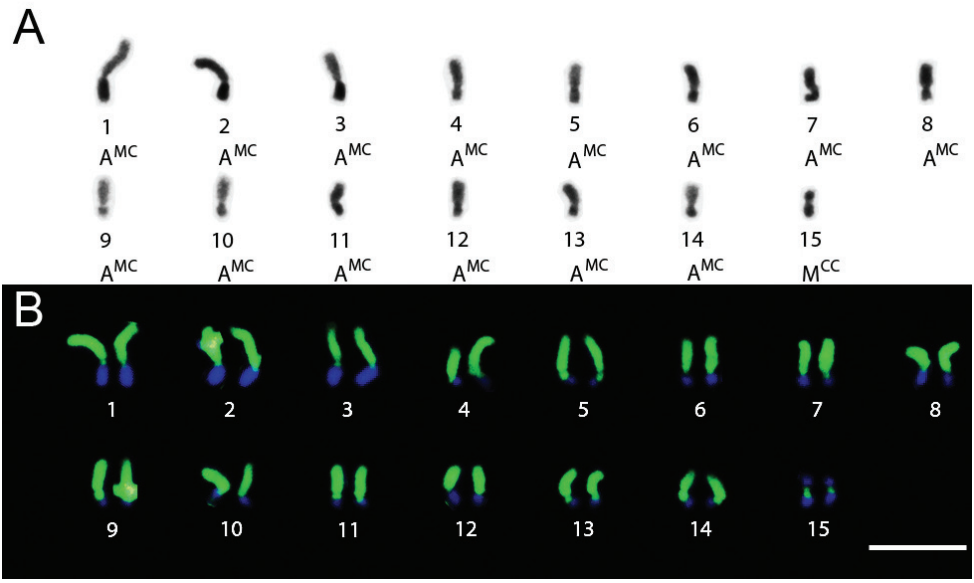

Figure 3A–B. *Trypoxylon* (*Trypargilum*) *lactitarse*. **A** Male karyotype (n=15) with standard staining **B** CMA_3/DAPI staining of female karyotype (2n=30). Bar=10μm.

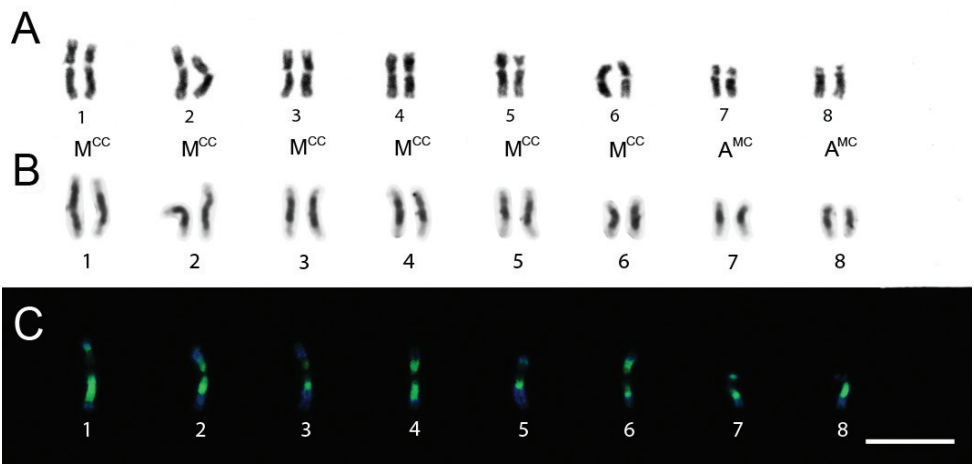

Figure 4A–C. *Trypoxylon (Trypoxylon) rugifrons*. **A** Female karyotype (2n=16) with standard staining **B** C-banding patterns in female karyotype (2n=16) **C** CMA_3/ DAPI staining of male karyotype (n=8). Bar=10μm.

The specimens of *T. nitidum* from Ilhéus with 2n=29 also had a small metacentric chromosome (15th pair) with a GC-rich arm whose homologue was not present. These results have also revealed band similarities between the GC-rich arm and the terminal region of the 1st pair, which is also GC-rich (Fig. 2B). This evidence leads us to infer that a fusion between the 15^{th} pair and the terminal region of one of the chromosomes of pair 1 could be involved in this numeric variation. This could also explain the het-

eromorphism of a CMA_3^+/$DAPI^-$ block present in one of the homologues of the 1st pair (Fig. 1B). Araújo et al. (2000) verified that the B chromosome in *Trypoxylon albitarse* was also stained by CMA_3, similarly to the constitutive heterochromatin of all chromosomes in the complement, thus suggesting that the B chromosome originated from breaks in the constitutive heterochromatin and is maintained in the genome by centromeric "reactivation". Scher and Pompolo (2003) did not report *T. nitidum* specimens with 2n=29 in a population collected at the Parque Estadual do Rio Doce, in the state of Minas Gerais (19°30'S, 41°1'W). This heterogeneity in karyotype over the distribution range highlights the need for further investigations in different localities to better resolve both karyotype distribution mapping and variation among *T. nitidum* populations. Further investigation will also be useful to confirm or reject whether the variable chromosomes are accessory in *T. nitidum*.

The heteromorphism found in the 5th pair in *T. nitidum* and in the 4th pair in *T. lactitarse* could be explained by a deletion or, alternatively, by a tandem growth of the heterochromatic blocks in the long arms. A previous study by Gomes et al. (1997) also found heteromorphism in *T. nitidum*, which those authors attributed to a deletion in one of the homologues. Based on the current knowledge on this matter, we consider both possibilities equally likely.

Several studies have demonstrated a correspondence between CMA_3^+ bands and rDNA sites in species such as *Scaptotrigona xanthotricha* Moure, 1950 and *Melipona* Illiger, 1806 bees (Rocha et al. 2002, Duarte et al. 2009), *Donax trunculus* L., 1758 (Mollusca: Bivalvia) (Petrović et al. 2009) and *Citrus* L., 1753 plants (Silva et al. 2010). It is therefore reasonable to predict that the CMA_3^+/$DAPI^-$ block in the terminal region of the 1st pair in all specimens of *T. nitidum* may bear rDNA. The absence of a CMA_3^+/$DAPI^-$ block in *T. lactitarse* and *T. rugifrons* in euchromatic regions may be attributed to differences in the molecular composition of the rDNA. Using fluorescent *in situ* hybridization with an rDNA probe in *T. albitarse*, Araújo et al. (2002) found that all rRNA genes are located on the heterochromatic arm in a single chromosome pair.

The three species studied herein are similar in their heterochromatin composition. However, the heterochromatin of the pseudoacrocentric chromosomes in *T. nitidum* has a balanced GC and AT composition in contrast with the acrocentric and metacentric chromosomes, which heterochromatin is GC-rich. This result indicates a difference in the heterochromatin composition in *T. nitidum*. Intraspecific variation in heterochromatin base composition has already been detected in other studies. For example, Domingues et al. (2005) detected a single chromosome pair with GC-rich heterochromatin in contrast with all the remaining AT-rich chromosomes in *Trigona fulviventris* Guérin, 1835 using C-banding and fluorochrome staining. Together with the previously described karyotypes, our results document a high frequency of large pseudoacrocentric chromosomes in Neotropical *Trypoxylon* species. These results lead us to believe that, regardless of base composition of the heterochromatin, the process of heterochromatin amplification has played an important role in the karyotype evolution of Neotropical fauna of aculeate Hymenoptera.

The euchromatin was AT-rich in the three species studied, which agrees with Araújo et al. (2000) who also found AT-rich euchromatin in *T. albitarse*. Our results indicate a high conservatism of the euchromatic regions as opposed to the heterochromatic regions that have a high rate of change. Repetitive segments such as the heterochromatin are considered hot spots of chromosomal rearrangements (Eichler and Sankoff 2003). Due to the high heterochromatin content, it is particularly likely that these species are undergoing a process of rapid genome reorganization that manifests itself through the chromosome variation and heteromorphisms found in this study. Our results, as well as previously published studies show a high karyotype variation in *Trypoxylon*. However, the genus is still poorly known and lacks a taxonomic reevaluation under a phylogenetic approach that could allow robust assumptions to be drawn on the pattern of its chromosome evolution.

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RESEARCH ARTICLE

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Banded karyotype of the Konya wild sheep (*Ovis orientalis anatolica* Valenciennes, 1856) from Turkey

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Abstract

The karyotype, C-banding, and nucleoar organizer regions (NORs) of eight specimens of Konya wild sheep from Turkey were examined. The complement included six large metacentric autosomes, 46 acrocentric autosomes of decreasing size, a medium-sized acrocentric X chromosome, and a small bi-armed Y chromosome (the diploid chromosome number 2n=54, the number of autosomal arms NFa=58, the number of chromosome arms NF=61). G-banding allowed reliable identification of all the chromosome pairs and the pairing of homologous elements. All the autosomes possessed distinct centromeric or pericentromeric C-positive bands. The X chromosome had a pericentromeric C-positive band, and the Y chromosome was entirely C-heterochromatic. The NORs were located in the terminal regions of the long arms of three metacentric and two acrocentric autosomes. The karyotype of the Konya wild sheep and its banding patterns are quite similar to chromosome complement reported in domestic sheep and European mouflon.

Keywords

chromosomes, *Ovis*, sheep domestication, Turkey

Introduction

The systematics of the genus *Ovis* (Linnaeus, 1758) is rather complicated and there are different opinions in respect of the number of species and the actual species borders (Nadler et al. 1973a, b, Shackleton et al. 1997, Grubb 2005). One of the main problems is the origin and taxonomic position of the domestic sheep. For the domestic sheep a large number of possible ancestral species and subspecies exist, and several Eurasian wild sheep

taxa have been proposed as ancestors of domestic sheep or are believed to have contributed to specific breeds. Mouflons in the Mediterranean islands [*O. musimon* (Pallas, 1811); *O. ophion* Blyth, 1841] introduced to various parts of Europe are generally evaluated as feral populations of ancient domestic stocks (e.g. Hiendleder et al. 1998a, b). Phylogenetic analyses on mtDNA restriction fragment length polymorphism of Eurasian breeds of domestic sheep demonstrated two maternal origins among modern sheep breeds and indicated wild ancestors different from the previously considered urial (*O. vignei* Blyth, 1841) and argali sheep [*O. ammon* (Linnaeus, 1758)] (Hiendleder et al. 1998b). In a subsequent study, Hiendleder et al. (2002) confirmed two well-separated mtDNA lineages (European and Asian) suggesting domestication from two distinct wild populations.

The domestic sheep and its wild ancestors are sometimes considered conspecific, in which case the name *O. orientalis* has generally been used, although some authors use the name *O. aries* for both wild species and its domestic descendants (Grubb 2005). Likely candidates for wild ancestors of the European lineage of domestic sheep are populations found in Turkey and western Iran. At the species or subspecies level, these populations are currently referred to *O. orientalis*, *O. orientalis* Gmelin, 1774, *O. gmelinii* Blyth, 1841, *O. anatolica* Valenciennes, 1856, *O. ophion* or *O. musimon* (Kaya et al. 2000, 2004, Kryštufek and Vohralík 2009). The Konya wild sheep is a westernmost taxon of extant wild sheep endemic to Central Anatolia in Turkey, and it is usually referred to *Ovis orientalis anatolica* (Kryštufek and Vohralík, 2009) or *O. gmelinii anatolica* (Kaya et al., 2000). The only population is currently living in the area east of Konya (Bozdağ Protection of Wildlife). The Konya wild sheep was widespread 50 years ago, but the numbers have decreased rapidly because of harsh weather conditions, predator pressure, and hunting. The most severe population decline appeared in 1960s and 1970s when the population size of 30–40 individuals was reported (Kaya 1991, Kaya et al. 2000, Sezen et al. 2004). The abundance has later increased owing to protection, and the current numbers are estimated at several hundreds of heads (600 individuals reported in 2006; Yalçın et al., 2010). The apparently last surviving population inhabits a large protected area surrounded with a fence providing open steppe habitats in altitude ranging between 1000–1735 m above sea level. The Konya wild sheep is not phenotypically akin to the European or Mediterranean mouflons, because it displays the type of horn configuration typical of Asiatic wild sheep (Yalçın et al. 2010).

Chromosome research has contributed significantly to understanding of the evolutionary divergence among sheep taxa, and different chromosome numbers were found in individual geographic populations. The European mouflon and wild sheep from western Palaearctic (*O. orientalis*) have the same diploid number of 54 chromosomes as the domestic sheep. In the Iranian populations of urial sheep the diploid number of 58 chromosomes was recorded, and hybrid zones with polymorphic karyotype were found in the areas of contact with *O. orientalis* (Nadler et al. 1973a, Valdez et al. 1978). The argali sheep possess 56 chromosomes in their complements, 2n=52 was found in *O. nivicola* Eschscholtz, 1929, and 2n=54 in both the American species (Vorontsov et al. 1972a, b, Nadler et al. 1973a, b, Korobitsyna et al. 1974, Bunch et al. 1976, 1998,

2000, Lyapunova et al. 1997). The chromosomal findings support the view that wild sheep of the urial or argali types did not contribute to domestication of the sheep. However, despite the differences in chromosome number, different species of the genus can hybridize in captivity (Vorontsov et al. 1972b, Nadler et al. 1973b).

The karyotype of the Konya wild sheep was studied by Kaya et al. (2004), and the diploid number of 54 chromosomes was ascertained. Only a description of the basic karyotype characteristics was given, without any illustrations. Subsequently, Kırıkçi et al. (2003) reported the G-banding pattern of chromosomes of this population. In the present paper, we provide detailed description of the karyotype of the Konya wild sheep, a possible ancestor of domestic sheep, using various chromosome banding techniques.

Material and methods

We examined eight specimens of Konya wild sheep including four females and four males. All the studied animals originated from Bozdağ in the Konya province, Turkey (Fig. 1). Chromosomal preparations were made using peripheral blood culture for 72 hr at 37°C in medium supplemented with 15% fetal calf serum and 0.024 % phytohaemagglutinin (Freshney 1990). The chromosome slides were conventionally stained with Giemsa. G-banding by trypsin treatment stained with Giemsa (GTG) was performed (Seabright 1971). Constitutive heterochromatin and nucleolus organizer regions (NORs) were detected with C-banding (Sumner 1972) and Ag-NOR staining (Howell and Black 1980), respectively.

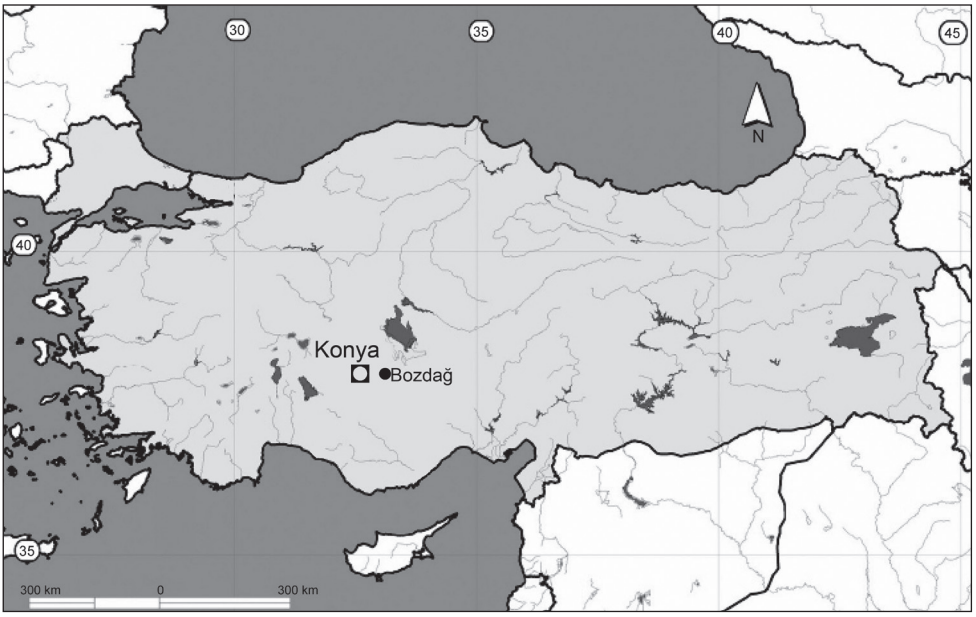

Figure 1. Collecting locality in the province of Konya, Anatolia, Turkey (●).

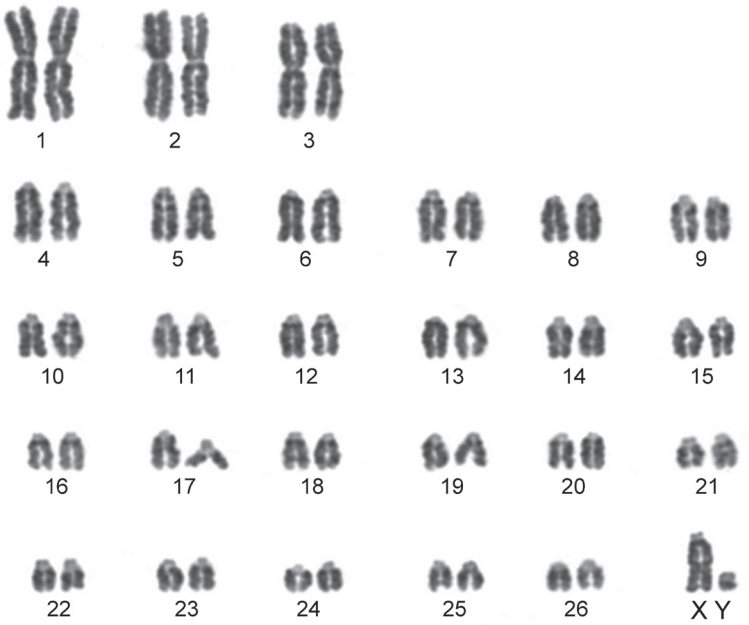

Figure 2. Conventionally stained karyotype of the Konya wild sheep with 2n=54. There are three pairs of large metacentric autosomes and 23 pairs of acrocentric autosomes. The X chromosome is acrocentric and the Y chromosome metacentric.

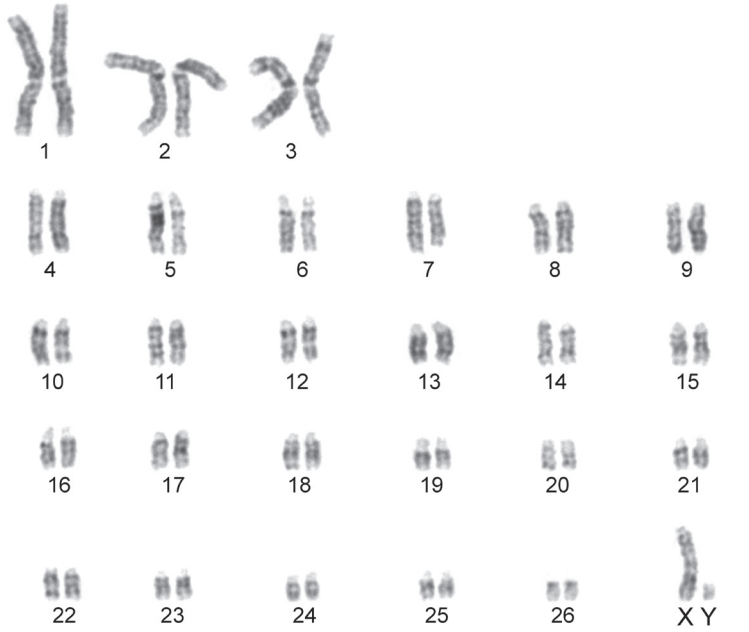

Figure 3. G-banded karyotype of the Konya wild sheep. All the chromosomes in the complement can be identified and differentiated according to their G-banding pattern.

Results and discussion

The karyotype contained 54 chromosomes (Fig. 2) including three large metacentric autosomal pairs (no. 1–3) and 23 acrocentric autosomal pairs of decreasing size (nos. 4–26; NFa=58). Tiny short arms were observed in most of the acrocentric autosomes. The X chromosome was a large acrocentric with a distinct short arm, whereas the Y chromosome was metacentric and the smallest element of the complement (NF=61). All the autosomes and both the sex chromosomes were reliably identified on the basis of their unique G-banding patterns (Fig. 3). The C-banded karyotype of Konya wild sheep is illustrated in Fig. 4. All the autosomes possessed distinct and large centromeric C-positive bands which usually extended in the neighbouring pericentromeric areas. The X chromosome had a pericentromeric C-positive band, and the Y chromosome was entirely heterochromatic. Silver-nitrate staining revealed that NORs were localized in the distal position on the long arms of three large metacentric (no. 1–3) and two acrocentric (nos. 4 and 8) autosomes (Fig. 5). All the NORs were observed in both the homologous chromosomes, except those of the pair no. 8 in which a heterozygous condition with the signal present in only one homologue was recorded in four specimens.

The obtained karyotypic data confirm possible close relationships between the Konya wild sheep and domestic sheep. The diploid number of chromosomes and their morphology expressed in the number of chromosomal arms and the proportion of the metacentric and acrocentric autosomes are the same in both complements. The detailed structure of banded chromosomes of the Konya wild sheep appears quite similar in comparison with the standard chromosomal complement of the domestic sheep (cf. Ansari et al. 1999; ISCNDB 2000). The G-banding pattern observed in individual autosomes and the sex chromosomes is apparently identical between the karyotype of the Konya wild sheep and domestic sheep. The amount of pericentromeric C-heterochromatin is noticeably large in both the complements, and the Ag-NOR sites are localized in the same homologous chromosomes. Heteromorphism in the number of active NORs found in the acrocentric autosomal pair no. 8 may have resulted from the small sample size used in this study.

A considerable degree of similarity can also be demonstrated by comparison of our results with previously reported differentially stained chromosomes of various wild sheep taxa (Nadler et al. 1973b, Valdez et al. 1978, Bunch et al. 1998, 2000, Kırıkçi et al. 2003). Banding pattern in chromosomes and the distribution of NORs are also quite similar to that reported for European mouflon (Slavíčková et al. 1985). We may conclude that the chromosomal data support the possible position of the Konya wild sheep as one of the ancestors of domestic sheep. The surviving population should be subjected to further extensive genetical research, and its strict protection should be maintained.

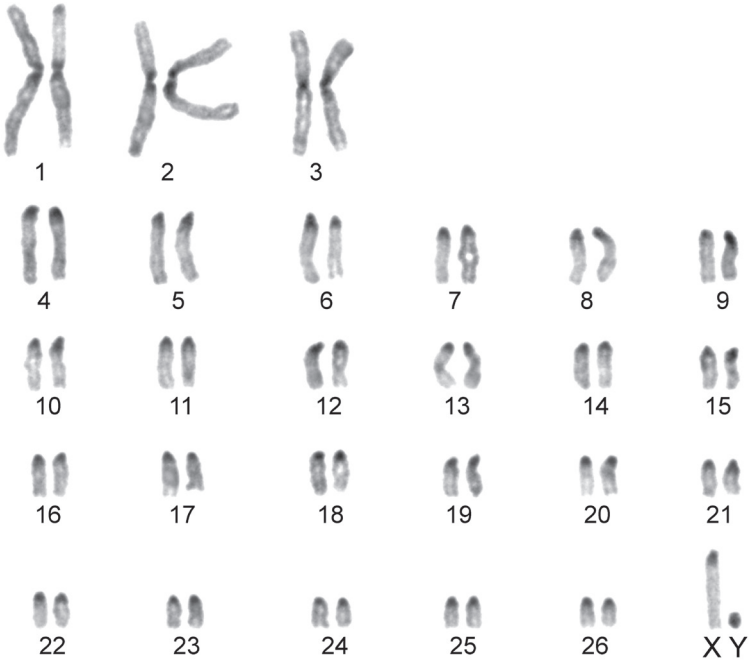

Figure 4. C-banded karyotype of the Konya wild sheep. Distinct pericentromeric dark bands are apparent in all the autosomes and the X chromosome. The Y chromosome is stained entirely positively.

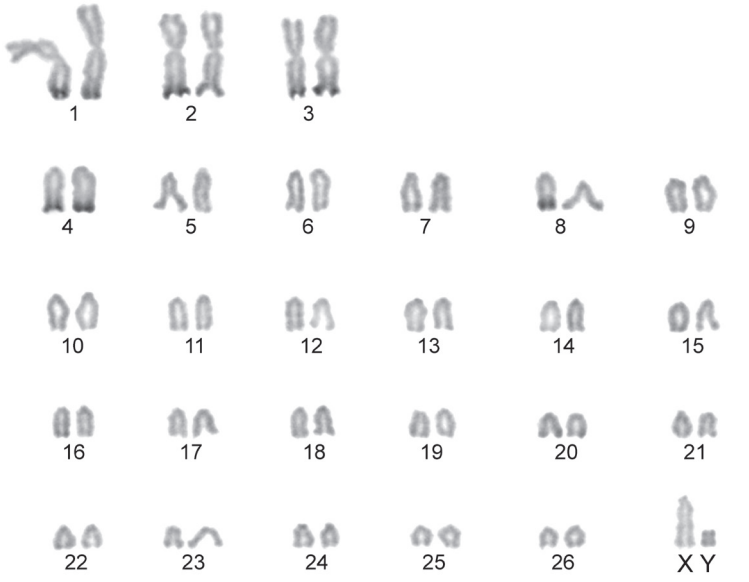

Figure 5. Silver-stained karyotype and the distribution of NORs in the Konya wild sheep. The NORs are localized in telomeric areas of the long arms of the three metacentric autosomal pairs (nos. 1, 2, 3) and two acrocentric autosomal pairs (nos. 4, 8). The heterozygous condition of the NORs in the acrocentric pair no. 8 was found consistently in various cells and different examined individuals.

Acknowledgments

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RESEARCH ARTICLE

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Polyploid races, genetic structure and morphological features of earthworm *Aporrectodea rosea* (Savigny, 1826) (Oligochaeta, Lumbricidae) in Ukraine

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Abstract

Four chromosomal races (2n=36, 3n=54, 6n=108, 8n=144) and 96 clones have been revealed among 224 specimens of the earthworm *A. rosea* over the territory of Ukraine by means of karyological analysis and biochemical genetic marking. Each population has been showed by several clones at least; moreover the clones from different places have never been identical. The clones in the range of one population can be identified with the set of quantitative and qualitative parameters.

Keywords

earthworms, *Aporrectodea rosea*, genetic structure, polyploid races, morphology

Introduction

Nowadays scientists are deeply interested with polyploid complexes of wide-spread animal species as most of such complexes became model objects for various experimental studies. Such interest has been caused by the conversion of the typological conception of species into the evolutional one. One of such complexes is the *Aporrectodea rosea* (Savigny, 1826) species complex that is widely spread in Europe and in particular over the territory of Ukraine.

Karyological studies (Muldal 1952, Omodeo 1952, Vedovini 1973, Mezhzherin et al. 2007, etc.) have showed the apomictic (parthenogenetic) species represented by series of

heteroploid forms. These forms are considered to appear as a result of serial hybridization of several diploid species (Victorov 1993). As Vsevolodova-Perel and Bulatova (2008) reviewed, the populations of *A. rosea* have turned out to have diploid (2n=36) or polyploid chromosome sets (3n=54, 4n=72, 4n=74–86, 5n=90, 6n=108, 8n=144, 10n=160–174).

Though the apomictic species may be represented by heteroploid forms, the one may contain from ten to several hundreds of clones (genotypes) with one ploidy level that can be revealed by means of analysis of allozyme variability (Lokki and Saura 1980; Jaenike et al. 1982; Terhivuo and Saura, 2006). The origin of heteroploid forms may be connected with hybridization of different parental species, and forming of clones within one chromosomal level does with spontaneous mutation process. The hybrid nature and genetic mosaicism of apomictic forms stimulate great theoretical interest; though they reveal the problems in practical systematics as hybrids have intermediate morphology comparing to parental forms and mask the status of parental species. One should also consider the transgressive character of diagnostic features of differentiation of parental forms. As a result the analysis of morphological variability of genetically marked material parental species and hybrid forms appears to be necessary.

The structure of diploid-polyploid complex based on gene marking of *A. rosea* was studied for several areas of this prevailing species only. There is only Fennoscandia in Europe, where the study of genetic structure of *A. rosea* has been carried on (Terhivuo and Saura 1993, 1996, 2006). They have never carried on this earthworm over the territory of the Eastern Europe up to nowadays. Preliminary data from *A. rosea* Ukrainian populations (Garbar and Vlasenko 2007, Mezhzherin et al. 2007, Vlasenko et al. 2007) have showed that this species is represented by several chromosome races with different ploidy and its high clone variability let it consider one of the most variable taxa of earthworms. Thus further studying of this species genetic structure on the territory of Ukraine by means of biochemical gene marking and cytogenetic methods appears to be actual.

Materials and methods

The materials for the present study were collected in spring - autumn period of 2004 - 2008 using generally accepted procedure (Byzova et al. 1987). 224 specimens identified as *A. rosea* according to the tables of Perel (1979) and Vsevolodova-Perel (1997) were used for karyological investigation. 60 samples from the Ukraine have been examined (Fig. 1). For the analyses, the most representative were samples from O.V. Fomin Botanical garden, Kyiv and Vylkove town, Odessa region, placed in the centre and south of the country (sites 1 and 2 in Fig. 1, resp.). Most often considered in the text are samples from the east and west to Kiyv containing specimens from sites 3–5 (Chernigiv region) and 6–7 (Zhitomir region).

Electrophoretic variability of spectra of enzymes (aspartate aminotransferase (AST), malate dehydrogenase (MDH), nonspecific esterases (ES) and superoxide dismutase (SOD) in extracts from a caudal part of body was investigated by the method of PAG–electrophoresis in Tris-EDTA-borate buffer system (Peacock et al. 1965).

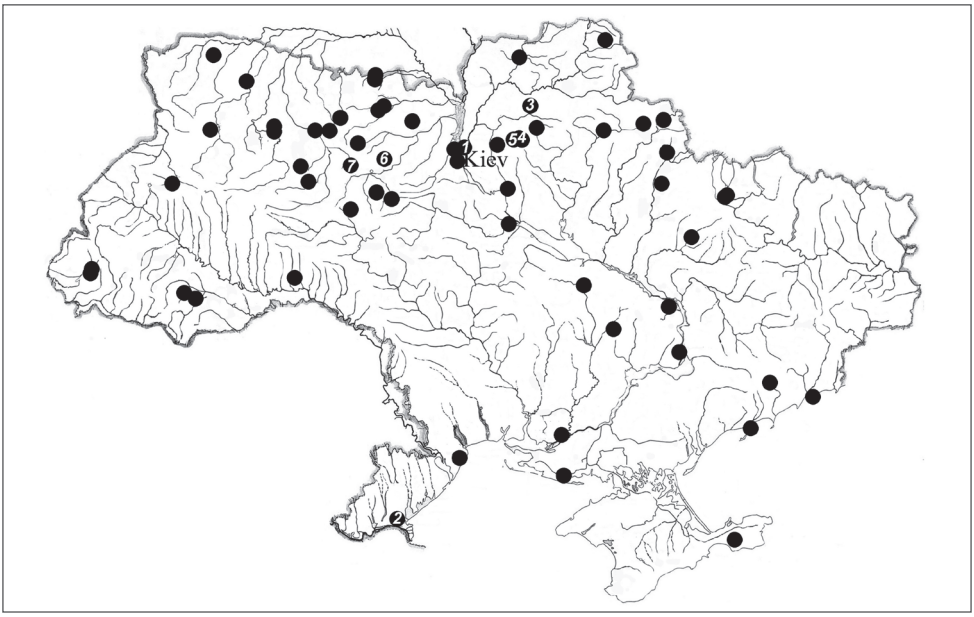

Figure 1. Localities of *A. rosea* samples in Ukraine. Numbers are given to the most representative samples studied in the centre of country (**1** Kiyv, state capital) and in the south (**2** Vylkove; Odessa region), east (**3** Nizhyn, **4** Novy Bykiv, **5** Serbi; Chernigiv region), west (**6** Zhitomir, **7** Romanov; Zhitomir region).

Chromosome preparations were made from seminal vesicles following the technique formerly used by the authors in Lumbricidae investigations (Garbar and Vlasenko 2007). The earthworms were injected with 0.1% colchicine 24 hours before the dissection. The spermatocytes were placed for 50 minutes in distilled water and fixed in the 1:3 mixture of glacial acetic acid and ethanol. Chromosome preparations were obtained by the reprint method (Sitnikova et al. 1990). Dried slides were stained with Giemsa-Romanovsky stain in 0.01M phosphate buffer (pH 6.8). The chromosome spreads were analysed with a «Mikmed» microscope (10×90).

The morphological studies have been started on the alive samples with defining the pigmentation of animal body and clitellum. The further studies have been held on the fixed earthworms when the body length (L) and clitellum (l1), the distance from the frontal part of the body to the clitellum (l2), the maximal diameter of the body out of the clitellum (d) have been measured. We have counted the quantity of segments (n1) and the quantity of segments up to the clitellum (n2), defined the head lobe form, the distance between the setae, the disposition of spinal pores and papilles, the size and position of clitellium, the form and position of pubertate platens by magnifying lens. Then the quantity of segments per 1 mm of body (n_1/L), the quantity of segments per 1 mm of body up to the clitellum (n_2/l_2), the comparative thickness of the body (l_2/d) have been measured. Statistical processing of obtained data was carried out by means of a package of applied statistical programs PAST 1.18.

Results and discussion

Biochemical gene marking

Nonspecific esterases. Nonspecific esterases of *A. rosea* are coded by more than four loci. Despite of the very high variability level with the quantity of fractions in one spectrum varying greatly it is difficult to show the proper quantity of loci for even one organism. The analysis of variability of this enzyme's spectrum proves the clone nature of variability by the fixation of the proper electrophoretic types. This way there the populations of reasonable variability (for example, the sample from O.V. Fomin National Botanical garden, Kyiv (Fig. 2a) with 16 electrophoretic spectra of nonspecific esterases have been found for 94 studied specimens), and populations with each specimen having its own type of the nonspecific esterases (cf. Novyi Bykiv and Galytsya) (Fig. 2b) have been found. We should point out at the fact that specimens with the identical spectra of nonspecific esterases have occured in one sample.

Aspartate aminotransferase. It was shown by series of alleles for this species. These series have single (homozygotic) or triple (constant heterozygotic) spectra typical for

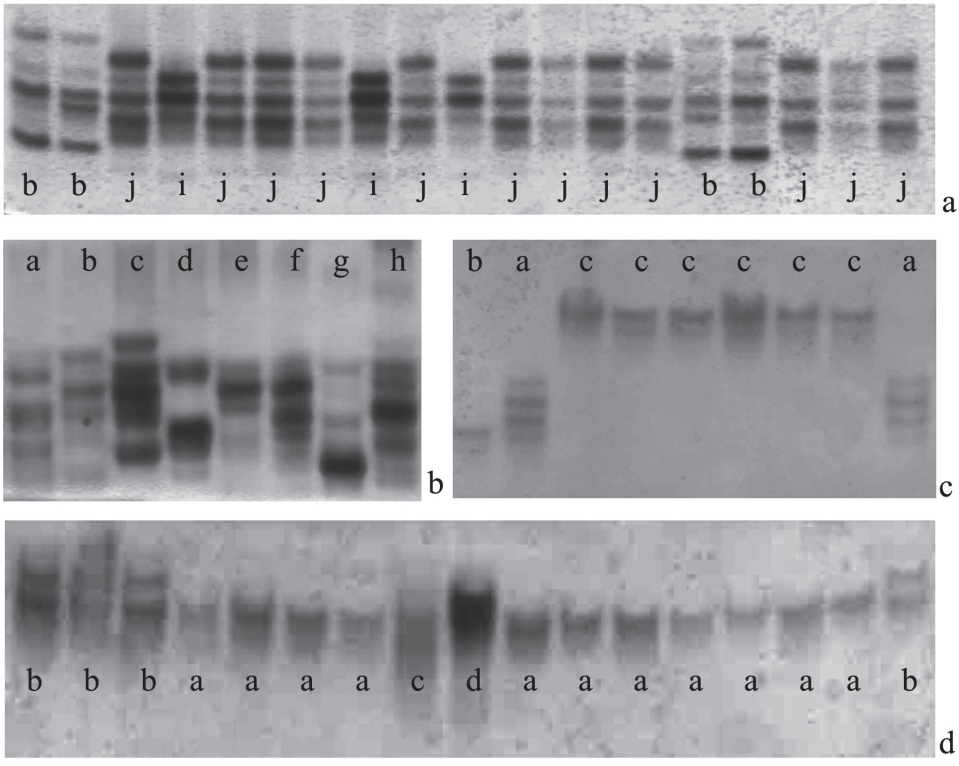

Figure 2. The geographic sample variability of *A. rosea* electrophoretic enzyme spectra: nonspecific esterases (**A** Kiyv; **B** Novy Bykiv); aspartate aminotransferase (**C** Vylkove); malat dehydrogenase (**D** Serbi); different clones are signed by letters.

dimerous protein (Fig. 2c). In the latter case they may have the effect of the gene dose. There have been 6 alleles found. Despite of the lower variability (comparing to the esterases' one) this enzyme and its coding loci have important diagnostic meaning for the clones' definition. It's worth pointing out the cases of finding the organisms with alternative alleles in one sample (Fig. 2c) as this fact reveals their independent origin and the absence of their alliances.

Malate dehydrogenase. This enzyme system is considered more conservative than aspartate aminotranspherase one. However sometimes it also shows the clone characteristics of the organism. Quite often heterozygotes have the asymmetric spectra (Fig. 2d) that points at the effect of the gene dose. There 5 alleles have been found.

Clone structure

The study of all over the territory of Ukraine. The 96 clones distributed over 60 localities examined have been defined by means of biochemical gene marking of 224 earthworms that have been defined as *A. rosea* due to their morphological features. This way more than half of them (67 clones) have been individual specimens that was considered to be normal for highly variable apomictic earthworms (Terhivuo and Saura 2006). The maximal quantity of organisms for one clone of 30 specimens has been found in season sample series in Botanical garden (Kyiv).

The average quantity of specimens for one clone has appeared to be only 2.33 ± 0.10 with the standard dispersion of σ=3.63 for all samples of *A. rosea.* The correlation of the average quantity and dispersion of this species proves the tendency of the organisms' random distribution as to the clones. Some tendency to the negative binominal distribution must be caused by the presence of unrandomising samples as the one from the botanical garden with its high number of organisms in one sample that's led to the unproportionally high clone representation of in this area. The estimation of biodiversity basing the Shannon-Weaver formula has showed the sample average index of 1.85 for samples of 5 and more specimens and the one of 5.72 for all the studied earthworms to characterize the high biodiversity. This fact gives the grounds for considering this species as so-called hypervariable taxon (Cywinska and Hebert 2002) being usual for small invertebrate agamospecies.

As most of the studied samples of the territory of Ukraine have been not numerous, the further discussion has been based on two most representative samples from Kyiv (site 1) and Vylkove (site 2).

The study of some settlements. The 13 clones have been defined by means of biochemical gene marking of 90 specimens of the *A. rosea* population of Kiyv of the spring-autumn period 2006 (Fig. 1, Table 1). It appears to be 6.9 ± 0.27 specimens per clone. Among them 6 (46%) clones have been shown to be individual specimens and 3 of them (23%) have had only two specimens. These way 4 dominant clones have got only 31% of their general quantity. Besides as to the quantity of organisms (78) they get up to 86.6% of the sample. The quantity of the found organisms varies from

Table 1. Genetic structure of *A. rosea* clones.

Kyiv					Vylkove				
Clone	**ES**	**AST**	**MDH**	**n**	**Clone**	**ES**	**AST**	**MDH**	**n**
A	a	b	c	29	A	a	b	a	13
B	b	a	a	30	C	b	c	a	9
B’	b’	a	b	2	A”	a	b	b	4
C	i	a	a	5	A’	b	b	d	1
D	j	a’	a	14	A”’	e	b	a	1
D”	d	a’	a	2	A””	e	e	?	1
E	d	b	a’	1	C’’	a	c	a	1
F	e	c	b	1	C”’	e	c	a	1
G	f	c	b	1	E	c	a	a	1
H	e	d	a	1	D’	d	b	a	1
I	c	a	a	2	F	t	b	a	1
K	h	a	a	1	B	c	d	c	1
L	i	e	c	1	D	a	a	?	1

5 (*A. rosea*-C) to 30 (*A. rosea*-B) in these clones’ measures. The distribution of these clones according to the quantity of specimens appears to correlate the distribution of rare events (Puasson distribution) that corresponds to stochastic laws. The close indexes of average (M=6.9) and standard dispersion (σ=7.78) point out at the same fact.

We have been able to observe the sudden changes in the clones structure of the different season samples (Table 2). This way it has been not only the rare clone structure that changes but the dominant one does as well (Fig. 3).

The 13 clones have been defined by means of biochemical gene marking of 36 specimens of the *A. rosea* population of Vylkove of the spring-autumn period of 2006 (Fig. 1, Table 1). It appeares to be 2.8 per clone. Among them 10 (76.9%) clones have been shown to be individual specimens and 3 dominant clones get 23.1% of their general quantity. Besides as to the quantity of organisms (26) they get up to 72.2% of the sample. The quantity of the found organisms varies from 4 (*A. rosea*-C) to 13 (*A. rosea*-B) in these clones’ measures. The distribution of these clones according to the quantity of specimens appears to correlate the Puasson distribution. The close indexes of average (2.76) and standard dispersion (3.83) point out at the same fact.

Table 2. The representation of *A. rosea* clones in the season samples from Kiyv.

Period of the specimens collecting	**clones**	**n**
June-July 2005	A, B, C, D’	9
August 2005	A	10
May-June 2006	A, B, D’	13
July 2006	A, B, I, J, K	29
September 2006	B, B’, C, D	25
October 2006	D	3

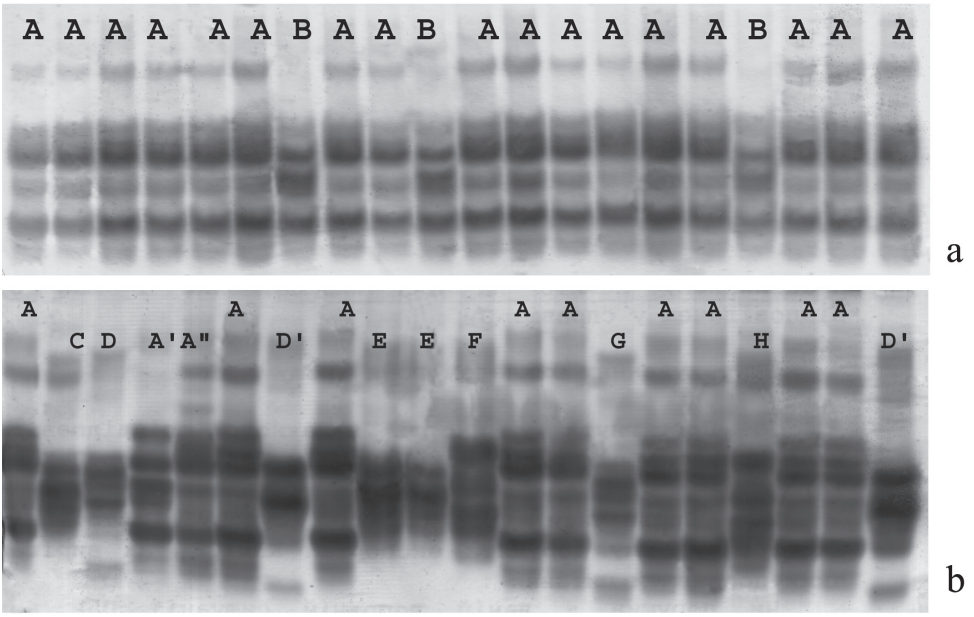

Figure 3. The seasonal variability of nonspecific esterases and clonal diversity of the *A. rosea* populations from the Kiyv sample: **a** June **b** October.

Karyological analysis. The specimens that could be used for karyological analysis have been obtained of only five *A. rosea* samples which, however, represent 4 different regions in the centre, east, west and south of Ukraine (Table 3). In the Kiyv sample, 33 mitotic metaphases of *A. rosea* 11 specimens were studied. We have also showed the *A. rosea*-A clone being characterized by triploid number of chromosomes 3n=34, NF=108 by analyzing five specimens. We have got 6 metaphase plates of two specimens of *A. rosea*-B clone with diploid number of chromososmes (2n=36, NF=72). This clone must be represented by diploid race. The clone *A. rosea*-C has been characterized by hexaploid number of chromosomes 6n=108, NF=216 (Fig. 4). The specimens of *A. rosea*-D clone has been characterized by octaploid number of chromosomes (8n=144). The chromosomes of the triploid race have been typically smaller than the ones of the diploid race.

In the Vylkove sample, we have got 20 mitotic metaphases of the five specimens of the most spread clone A. The organisms of this clone have been found to have triploid number of chromosomes: 3n=54, NF=108.

We have showed the triploid number of chromosomes 3n=54, NF=108 for samples from other regions, too. Eight specimens of Nizhyn sample, six specimens from Zhitomir and four from Romanov all were triploid and, above, one organism with hexaploid chromosome number have been found.

Thus, the series of heteroploid *A. rosea* clones from different places of Ukraine were found by means of karyological analysis (Table 3). With all this going on dominant number of chromosomes is shown to be triploid considered its being the most spread over the territory of Ukraine (Viktorov 1993).

Table 3. Karyotypes of *A. rosea* from Ukraine and their belonging to clones.

Sample (site on the map)	Clone	Karyotype	The quantity of plates	The quantity of specimens
Kiyv (1)	A	3n = 54	23	5
	B	2n = 36	6	2
	C	6n = 108	8	3
	D	8n = 144	4	2
Vylkove (2)	A	3n = 54	20	5
Nizhyn (3)	A	3n = 54	23	8
Zhitomir (6)	A	3n = 54	12	6
Romanov (7)	A	3n = 54	17	4
	B	6n = 108	2	1

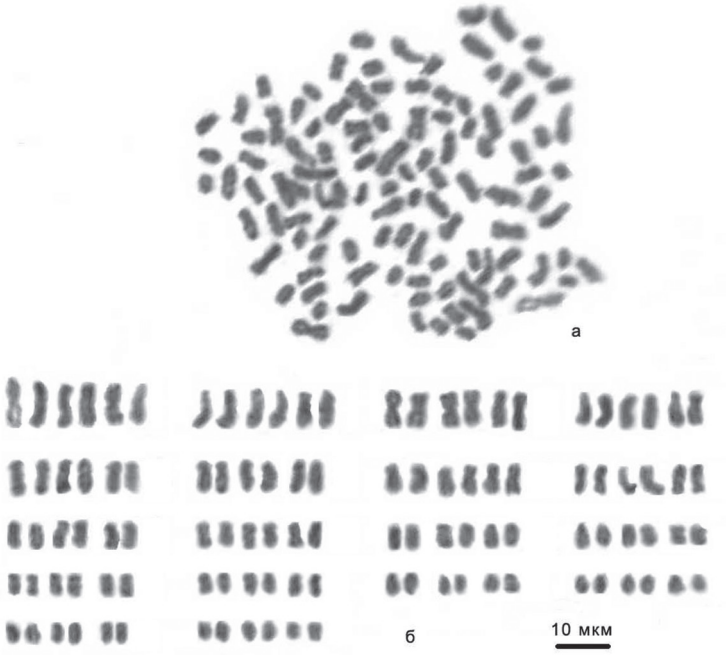

Figure 4. The hexaploid chromosome set (6n=108) of the clone *A. rosea*-C from Kiyv: **A** mitotic metaphase **B** karyogram.

The analysis of morphological features of different clones representatives. We have used the wide-spread *A. rosea* clones of earthworms from Kyiv and Vylkove for morphological analyses. We should point out that the analysis of different clones of the same residence let's avoiding the environmental effect that anyway leads to modificating the morphological appearance of earthworms in samples from different regions.

Kiyv. Average values of investigated parameters of four *A. rosea* wide-spread clones (A, B, C, D) are given in Table 4. As for the results of dispersive analysis (LSD–test), *A. rosea*-A clones (basing the absolute (D) and comparative (l_2/D) body diameter)

Table 4. Average (M) and standard errors (m) of morphological parameters of *A. rosea* clones. L - length of whole body, l_1- clitellum, l_2- forward end of body, D - maximal diameter of a body behind clittelum, n_1 - total number of segments, n_2 - number of segments up to clitellum.

Parameters		Clone /Number of specimens						
		Kiyv				Vylkove		
		A(29)	B(30)	C(5)	D(14)	A(13)	C(4)	A"(9)
L, MM	M	27,14	31	31,6	29,36	30,62	30,5	29,67
	m	1,26	0,88	1,12	1,09	1,49	1,04	2,86
n1	M	96,14	110,03	110,2	104,86	109,23	107,25	100,44
	m	3,52	2,39	3,32	2,39	4,95	5,3	10,02
n1/L	M	3,6	3,58	3,49	3,61	2,88	3,13	2,56
	m	0,06	0,05	0,02	0,08	0,23	0,13	0,23
n2/l2	M	2,86	2,85	2,87	2,98	7,5	7,25	7,72
	m	0,03	0,08	0,08	0,05	0,27	0,25	0,25
l2, mm	M	8,19	8,85	8,4	8,43	3,06	2,88	2,64
	m	0,05	0,32	0,24	0,14	0,13	0,13	0,13
l1, mm	M	3,28	3,09	3	2,93	2,47	2,53	2,96
	m	0,09	0,13	0,32	0,22	0,07	0,12	0,13
D, mm	M	2,15	1,92	1,7	1,71	3,6	3,53	3,38
	m	0,06	0,04	0,12	0,07	0,14	0,21	0,05
l2 /D	M	3,89	4,48	5	4,97	3,3	3,41	3,09
	m	0,06	0,11	0,2	0,11	0,12	0,1	0,11

and *A. rosea*-B clones (basing comparative body diameter) are well-differentiated ones for the morphological features, while no reliable difference between *A. rosea*-C and *A. rosea*-D clones have been found. The qualitative parameters also allow the reliable differentiating of most of clones (Table 5).

The discriminant analysis of the features' parameters has showed the studied clones to be discriminated better within the qualitative features but for the quantitative ones (97.44 and 61.54% respectively). Considering all the features even leads to some lowering of the discrimination accuracy (96.15%) comparing to the one based on the qualitative parameters.

The dispersion diagram (Fig. 5, a) shows only *A. rosea*-D standing out for the first discriminant function. *A. rosea*-A, *A. rosea*-B and *A. rosea*-C are differed with the second discriminant function. The using of other pairs of discriminant functions has given the same results.

Vylkove. We have used three most wide-spread clones (A, A", C) for morphological analysis. Average values of their investigated parameters are given in Table 4. The dispersive analysis (LSD–test) has showed the presence of their possible distinctions with some features. For example the *A. rosea*-A – *A. rosea*-A" clone pair differ significally with the absolute (D) and relative (l_2/ D) diameter of the body, though *A. rosea*-C

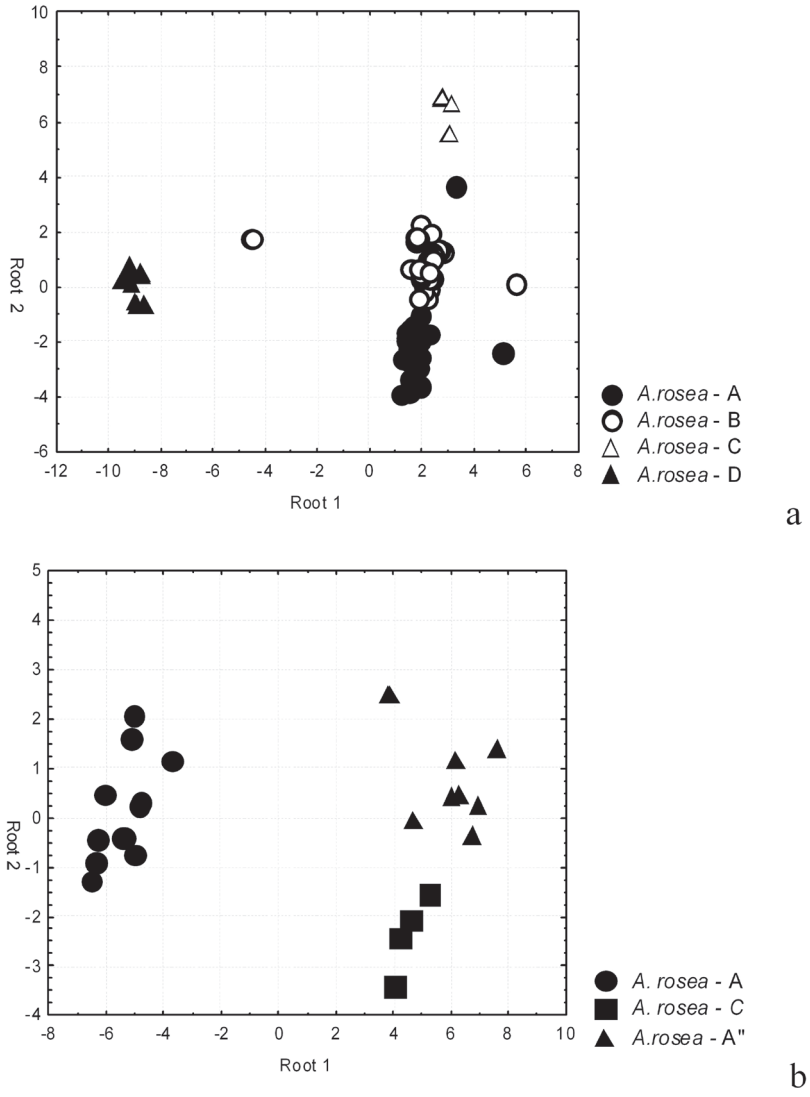

Figure 5. The distribution of the studied *A. rosea* samples in the zone of discriminant functions: **a** Kiyv (north) **b** Vylkove (south).

- *A. rosea*- A" does only with the relative one. Besides, the clones used for the analysis are reliably differentiated within the number of qualitative features (Table 5).

The discriminant analysis has showed all the studied clones to be discriminated well (100%) basing the set of features. The clone *A. rosea*-A stands out well with the first discriminant function (Fig. 5, b). *A. rosea*-A" and *A. rosea*-C are differed with the second discriminant function. Thus all three dominant clones are districted from each other rather well.

Table 5. Frequencies (%) of qualitative parameters of *A. rosea.*

Features	Variants of parameters	Kiyv				Vylkove		
		A	B	C	D	A	C	A"
		n= 29	n= 30	n= 5	n= 14	N=13	N=4	N=9
Beginning of clitellum (segments)	24	0	0	0	0	15.4	0	22.2
	25	100	100	100	0	15.4	25	77.8
	26	0	0	0	100	69.2	75	0
Ending of clitellum (segments)	31	3.5	0	0	0	7.7	0	0
	32	96.6	100	100	100	15.4	100	100
	33	0	0	0	0	76.9	0	0
Pigmentation of body	Unpigmented	0	0	0	0	100	100	66.7
	Light pink	100	100	100	100	0	0	33.3
Pigmentation of clitellum	Deep orange	96.6	0	0	100	100	0	0
	Light orange	0	100	0	0	0	0	22.2
	Pink and white	0	0	0	0	0	100	55.6
	Unpigmented	0	0	100	0	0	0	22.2
	Red	3.5	0	0	0	0	0	0

Conclusions

The incredibly high variability of *A. rosea* clones has been found out (96 clones per 224 studied specimens from 60 localities) in our study. As a rule, several clones have been studied in the range of one population and the clones of different places have never been identical ones. We have expected for such results basing the Fennoscandian data (Terhivuo and Saura 2006). Authors have managed to find 46 clones per 155 studied organisms that comes to 3.4 organisms per one clone. Here the high interclonal and geographical variability of morphological features has become apparent. According to our data the clone variety of this species is higher for the territory of Ukraine than for Fennoscandia and comes to 2.33 organisms per clone.

Such a high level of genetic variability of this species must be induced by the high genome mutability that must be caused the recombination of the genetic material that has led to forming of not only lot of genetic form but the heteroploid races as well. For example di- (2n=36), tri- (3n=54), hexa- (6n=108) and octaploid (8n=144) *A. rosea* specimens have been found on the studied territory. This way the triploids appear to be the most wide-spread ones and get nearly 73% of the general quantity of the studied specimens. The significant heterogeneity of this species distinctly correlates the high clone variability. We have managed to define the set of features for the clones' identification in the range of one population. They are districted typically better with their qualitative features than with the linear parameters ones. Besides, the clone structure of *A. rosea* population is characterized by distinct season variability that proves the ecological differentiation of clones. Further sampling is needed, however, to support the preliminary observations presented here.

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RESEARCH ARTICLE

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Karyotype analyses of ten sections of *Trigonella* (Fabaceae)

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Abstract

Karyotypes of ten sections of genus *Trigonella* Linnaeus, 1753 (Fabaceae) from Turkey were investigated. Somatic chromosome numbers of examined species were determined as 2n=14 and 16. The karyotype analyses of the species were carried out and somatic chromosome numbers of *Trigonella plicata* Boiss., 1872, *T. brachycarpa* (Fisch.) Moris, 1833, *T. rostrata* Boiss., 1872, *T. lunata* Boiss., 1843, *T. isthmocarpa* Boiss. et Balansa 1856, *T. rhytidocarpa* Boiss. et Balansa, 1859, *T. spicata* Sibth. et Sm., 1813, *T. cephalotes* Boiss. et Balansa 1856, *T. capitata* Boiss., 1843 and *T. gladiata* Steven, 1808 were reported for the first time. Two pairs of satellite metaphase chromosomes were observed in *T. cariensis* Boiss., 1843 and one pair in *T. lunata*. Moreover, 2 B-chromosomes were found only in *T. procumbens* Rchb., 1830 among all studied species. The aims of this study are to provide karyological data for a significant pool of the taxa, to show differences among them in the number, size and morphology of somatic chromosomes, to verify previous reports or represent numbers which are different from those cited previously.

Keywords

Somatic chromosome, Turkey, Fabaceae, karyotype

Introduction

Trigonella L., 1753 (Fabaceae) includes about 135 species worldwide, and most of the species are distributed in dry regions around Mediterranean, West Asia, Europe, North and South Africa, North America, and with only two species being present in

South Australia (Mabberly 1997). The genus *Trigonella* has 13 sections and 50 species in Turkey (Huber-Morath 1970). *Trigonella* species are localized in different phytogeographical regions in Turkey with 21 endemic species showing 42% endemism rate (Huber-Morath 1970, Martin et al. 2008).

According to the literature, some studies conducted on the karyology of the *Trigonella* include approximately hundred species (Darlington and Wylie 1955, Tutin and Heywood 1964, Ghosh 1980, Astanova 1981, Agarwal and Gupta 1983, Ladizinsky and Vosa 1986, Danin and Small 1989, Bal 1990, Kumari and Bir 1990, Bidak and Amin 1996, Pavlova 1996, Yılmaz and 2006, Martin et al. 2008). The somatic chromosome numbers of the genus *Trigonella* are reported as 2n=14, 16 and 18. In addition, some chemical, morphological and taxonomical studies were conducted on *Trigonella* species (Meusel and Jager 1962, Sırjaev 1935, Baum 1968, Small et al. 1981, Small et al. 1981, Classen 1982, Small 1988, Danin and Small 1989, Small and Jomphe 1989, Alhabori et al. 1998, Sheoran et al. 1999, Ram and Verma 2000, Murakami et al. 2000, Oncina et al. 2000, Sur et al. 2001, Sabir et al. 2002, Petropoulus et al. 2002).

In the present work we carried out a karyological study on 19 species of *Trigonella*, belonging to ten sections, collected from different regions of Turkey (Huber-Murath 1970).

Material and methods

Seedlings were collected between the years of 2002 and 2005 from natural habitats in different localities (Table 1). For karyotype analyses, root tips were obtained from seeds germinated in humidified Petri dishes at room temperature. Root tips were pretreated with α-monobromonaphthalene at 4 °C for 16 h and fixed in Carnoy's fixative for 24 h at 4 °C. Before staining, the material was hydrolyzed with 1N HCl for 13–15 minutes at room temperature. The chromosomes were stained with 2% aceto orcein and mounted in 45% acetic acid. Permanent slides were made by using the standard liquid nitrogen method and then examined under Olympus BX50 Photomicroscope using an oil immersion objective (100 X). Photographs were taken with the same microscope. Karyotype analyses were made by the use of an Image Analysis System (Bs200Pro).

Results

This study was carried out to analyse the karyotypes of 19 species, eight of which are endemic to Turkey, belonging to ten sections of the genus *Trigonella* in Turkey. These sections are: *Samaroideae* Boiss., *Pectinatae* Boiss., *Lunatae* Boiss., *Falcatulae* Boiss., *Reflexae* (Širj.) Vass., *Isthmocarpae* Boiss., *Uncinatae* Boiss., *Capitatae* Boiss., *Bieberstеinianae* (Širj.), and *Foenum-graecum* Ser. Cytological results obtained from our study are arranged based on the order in the Flora of Turkey (Huber-Morath 1969).

Table 1. Localities, collector name and numbers of studied *Trigonella* species.

Taxa	Locality	Collector name and number
Trigonella cretica	Burdur: Gölhisar-Dirmil 4. km, 30.05.2002, 900–950 m, conserved area	H.Akan 3480, M. Ekici
Trigonella plicata	Konya: Hadim-Konya 10. km, 17.07.2002, 1350 m, roadside	H.Akan 3789, Z.Aytaç, M.Ekici
Trigonella brachycarpa	Konya: Hadim-Konya 10. km, 17.07.2002, 1400 m, roadside	H.Akan 3786, Z.Aytaç, M.Ekici
Trigonella rostrata	Konya: East of Hadim-Karaman, 17.07.2002, 1250 m, roadside	H.Akan 3805, Z.Aytaç, M.Ekici
Trigonella lunata	Adana: Pozantı-Çiftehan 8.km, 08.06.2003, 852 m, stony places	H.Akan 4687, M.Ekici
Trigonella corniculata	Izmir: Bayraklı, 26.05.2002, 20–50m, meadows Muğla: Bodrum castle, 25.05.2002, 10 m, meadows	H.Akan 4616, M.Ekici H.Akan 3391, M.Ekici
Trigonella spinosa	Muğla: Marmaris-Datça 50. km, 06.05.2005, 5–10 m, maqius	H.Akan 5655, M.Ekici
Trigonella monspeliaca	Muğla: Dalaman, Ortaca district, 24.5.2002, 20 m, stony places Antalya: Exit of Antalya-Hafızpaşa, 23.05.2002, 750 m, maqius	H.Akan 3358, M.Ekici H.Akan 3327, M.Ekici
Trigonella isthmocarpa	Aksaray: 28 km E of Aksaray, Hasan mountain foots, 08.06.2003, 1145 m	H.Akan 4695, M.Ekici
Trigonella rhytidocarpa	Niğde: Ulukışla-Pozantı 5. km, 16.07.2002, 1300 m, roadside	H.Akan 3778, M.Ekici
Trigonella spicata	Antalya-Bucak 20.km, Pınarbaşı district, 23.05.2002, 220–250 m, opening of *Quercus coccifera*	H.Akan 3319, M.Ekici, Karavelioğulları
Trigonella cephalotes	Aydın: Dilek Peninsula, National park, Aydınlık cove, 26.02.2002, 70 m	H.Akan 3398, M.Ekici
Trigonella procumbens	Konya: Akşehir-Gelendost 1–2.km, 22.06.2003, 1110 m, roadside of vineyards	H.Akan 4760, M.Ekici
Trigonella capitata	Denizli: Pamukkale, 22.06.2003, 1550 m, opening of damaged forest	H.Akan 4767, M.Ekici
Trigonella coerulescens	Konya: Ereğli-Karapınar 20–25km, 01.06.2002 1000 m, steppe Kayseri: Kayseri, Hisarcık, Erciyes mountain road, 08.07.2002, 1550 m	H.Akan 3587 Z.Aytaç, M.Ekici H.Akan 3659, M.Ekici
Trigonella gladiata	Karaman: Pınarbası-Kızılkaya 2 km, 28.06.2005, 1200 m, opening of Oak	H.Akan 5743, M.Ekici
Trigonella cariensis	Izmir: Ayrancılar-Izmir 6.km, 25.05.2003, 100 m, foots of maqius Antalya: Antalya-Hafızpaşa-Bucak 5.km, 23.05.2002, 775 m	H.Akan 4620, M.Ekici H.Akan 3332, M.Ekici
Trigonella foenum-graecum	Adana: Ceyhan-Adana 13.km., 20.05.02, 120 m, fields	H.Akan 3274
Trigonella macrorrhyncha	Içel: Tarsus-Çamlıyayla road, 30.km, 18.05.2003, 850 m, steppe	H.Akan 4354, M.Ekici

Section: *Samaroideae*

Trigonella cretica (L.) Boiss., 1872, 2n=16 (Fig. 1, A). All chromosome pairs at the somatic metaphase phase were observed metacentric. The chromosome length ranges between 0.52 and 1.01 μm. The total haploid chromosome length was measured as 5.88 μm.

Section: *Pectinatae*

Trigonella plicata (Boiss. et Bal.) Boiss., 1872, 2n=14 (Fig. 1, B). The species has metacentric chromosome pairs at somatic metaphase. The chromosome length ranges from 0.84 to 1.34 μm with the total haploid chromosome length of 7.12 μm. This species is endemic of Turkey.

Section: *Lunatae*

Trigonella brachycarpa (Fisch.) Moris, 1833, 2n=16 (Fig. 1, C). *T. rostrata* (Boiss. & Bal.) Boiss., 1872, 2n=14 (Fig. 1, D). *T. lunata* Boiss., 1843, 2n=16 (Fig. 1, E). All of the species of this section examined have metacentric chromosome pairs at somatic metaphase plates. For *T. brachycarpa*, chromosome length ranges from 0.94 to 1.36 μm having total chromosome length of 8.85 μm. *T. rostrata* being endemic to Turkey was observed having chromosomes whose lengths range between 0.81 and 1.22 μm. In this species, the total haploid chromosome length was measured as 7.07 μm. For the species *T. lunata*, the chromosome length was measured between 2.85 and 3.92 μm with the total haploid chromosome length of 26.44 μm. In addition, this species has a pair of satellite metaphase chromosomes.

Section: *Falcatulae*

Trigonella corniculata L., 1759 from the specimen collected from İzmir province; 2n=16 (Fig. 1, F) and from the specimen collected from Muğla province; 2n=16 (Fig. 1, G). At somatic metaphase, seven pairs of metacentric and one pair of submetacentric chromosomes were observed in the former while four pairs of metacentric and four pairs of submetacenric chromosomes were detected in the latter. The specimen collected from İzmir province has chromosomes ranging between 0.61 and 0.88 μm, and the total haploid chromosome length for this specimen was measured as 5.71 μm. In the other specimen which was collected from Muğla province, the chromosome length ranges between 1.54 and 2.57 μm with the total haploid chromosome length of 17.58 μm. *T. spinosa* L., 1753, 2n=16 (Fig. 1, H). At somatic metaphase, two pairs of metacentric and six pairs of submetacentric chromosomes were observed.

For this species, the chromosome lengths range from 1.49 to 2.76 μm with the total haploid chromosome length of 16.76 μm.

Section: *Reflexae*

Trigonella monspeliaca L. 1753 from the specimen collected from Muğla province; 2n=16 (Fig. 1I) while the specimen collected from Antalya province; 2n=16 (Fig. 1, J). In the former, all chromosome pairs at the somatic metaphase were observed to be metacentric, and the chromosome length ranges between 0.66 and 1.18 μm with the total haploid chromosome length of 6.81 μm. In the latter specimen, five pairs of metacentric and three pairs of metacentric chromosomes were observed at somatic metaphase. The chromosome length ranges between 0.74 and 1.00 μm. The total haploid chromosome length was measured as 7.06 μm.

Section: *Isthmocarpae*

Trigonella isthmocarpa Boiss. et Bal., 1856, 2n=16 (Fig. 1K) and *T. rhytidocarpa* Boiss. & Bal. 2n=16 (Fig. 1L). Endemic to Turkey, both species have metacentric chromosome pairs at the somatic metaphase. For the species of *T. isthmocarpa*, the chromosome length ranges between 0.57 and 1.10 μm. The total haploid chromosome length was measured as 6.53 μm. For *T. rhytidocarpa*, the chromosome length ranges between 0.85 and 1.40 μm with the total haploid chromosome length of 8.47 μm.

Section: *Uncinatae*

Trigonella spicata Sibth. et Sm., 1813, 2n=16 (Fig. 1M) and *T. cephalotes* Boiss. et Bal., 1856, 2n=16 (Fig. 1, N). At somatic metaphase, five pairs of metacentric and three pairs of submetacentric chromosomes were observed for both species. The chromosome length of *T. spicata* ranges between 1.00 and 1.50 μm with the total haploid chromosome length of 10.36 μm. For *T. cephalotes*, the chromosome length ranges between 0.43 and 0.94 μm. The total haploid chromosome length was measured as 4.49 μm. This species is endemic to Turkey.

Section: *Capitatae*

Trigonella procumbens (Besser) Reichp., 1830, 2n=16 (Fig. 1O) and *T. capitata* Boiss., 1843, 2n=16 (Fig. 1, P). Both species have metacentric chromosome pairs at the somatic metaphase. Endemic to Turkey, *T. procumbens* have chromosomes ranging from 0.82 to 1.32 μm with the total haploid chromosome length of 7.75 μm.

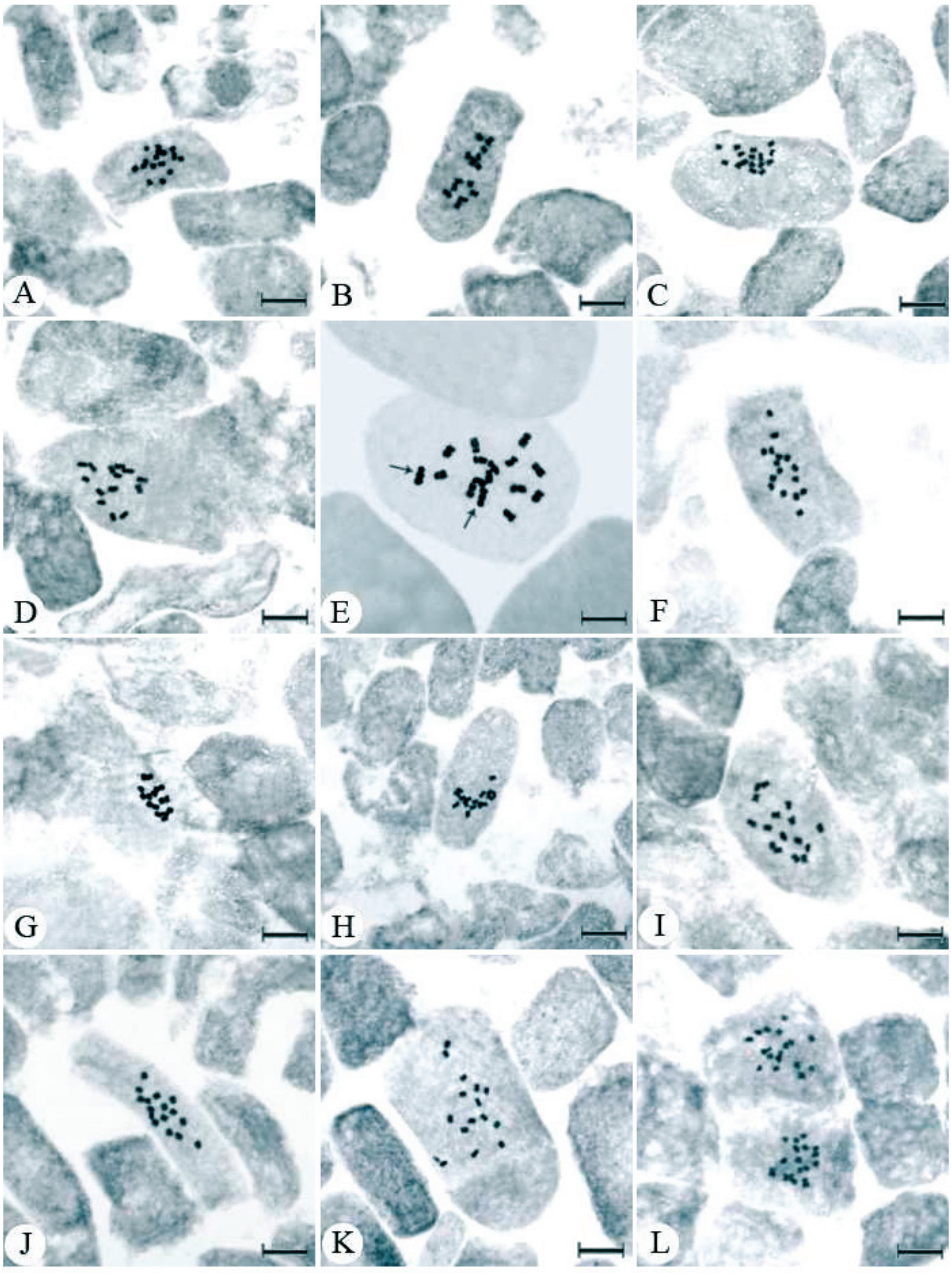

Figure 1. A–L Mitotic metaphase chromosomes of *Trigonella* species **A** *T. cretica* **B** *T. plicata* **C** *T. brachycarpa* **D** *T. rostrata* **E** The satellite (arrow) of chromosomes *T. lunata* **F** *T. corniculata*; no: 4616 **G** *T. corniculata* no: 3391 **H** *T. spinosa* **I** *T. monspeliaca* no: 3358 **J** *T. monspeliaca* no: 3327 **K** *T. isthmocarpa* **L** *T. rhytidocarpa*. Scale bar = 10 μm.

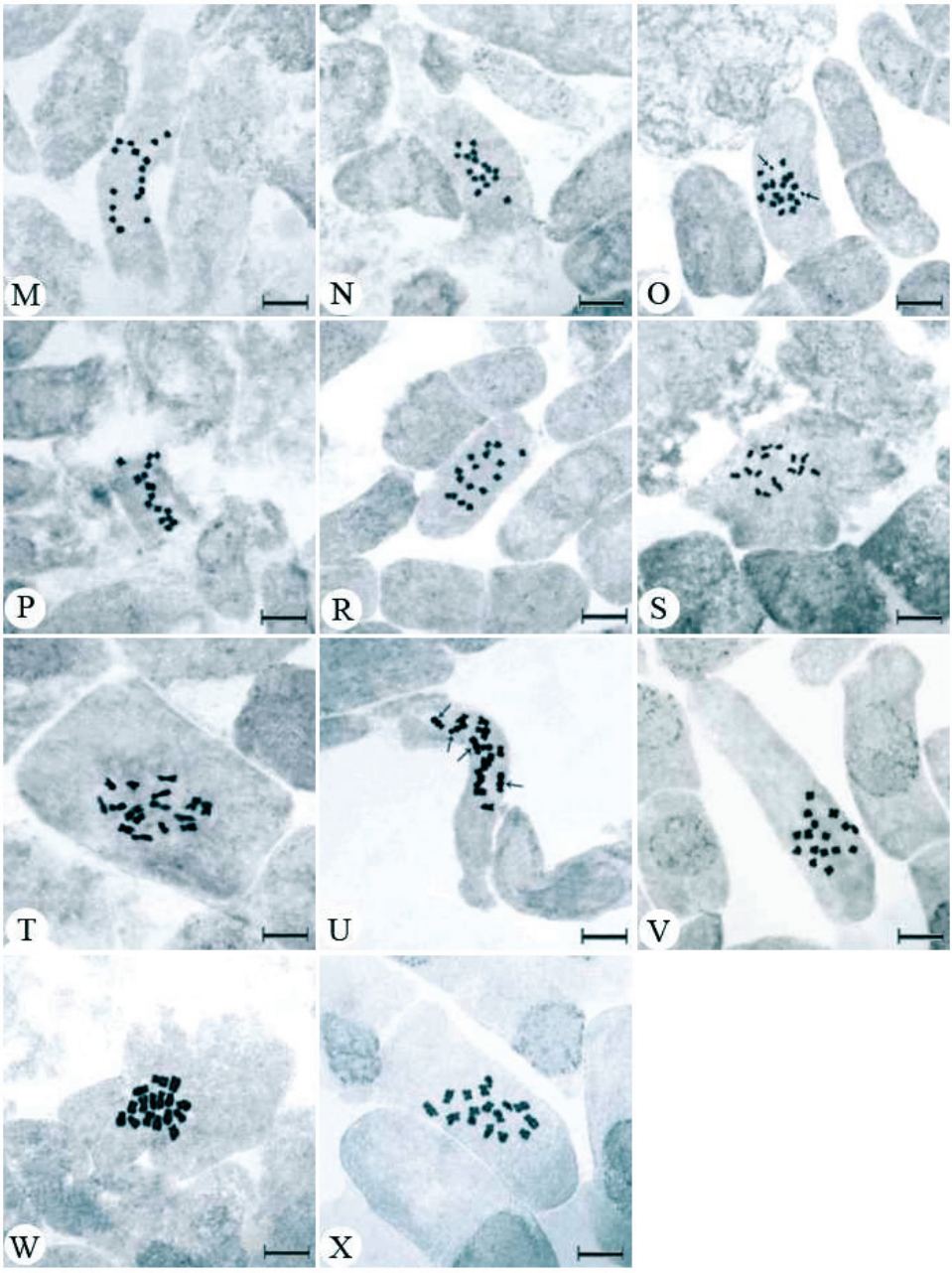

Figure 1. M–X Mitotic metaphase chromosomes of *Trigonella* species **M** *T. spicata* **N** *T. cephalotes* **O** B chromosomes (arrow) *T. procumbens* **P** *T. capitata* **R** *T. coerulescens* no: 3587 **S** *T. coerulescens* no: 3659 **T** *T. gladiata* **U** The satellite (arrow) of chromosomes *T. cariensis* no: 3332 **V** *T. cariensis* no: 4620; **W** *T. foenum-graecum* **X** *T. macrorrhyncha*. Scale bar = 10 μm.

Moreover, two B chromosomes are observed in this species. The other species of the section examined, *T. capitata*, have chromosomes ranging from 0.96 to 1.30 μm. The total haploid chromosome length was measured as 9.33 μm. This species is also endemic to Turkey.

Section: *Biebersteinianae*

Trigonella coerulescens (Bieb.) Hal., 1901, in the specimen collected from Konya province; 2n=16 (Fig. 1, R) and in the specimen collected from Kayseri province; 2n=16 (Fig. 1, S). All chromosome pairs at the somatic metaphase phase were observed to be metacentric for both specimens. The chromosome length ranges between 1.38 and 1.91 μm for the former while that of the latter ranges between 0.91 and 1.32 μm. The total haploid chromosome length was measured as 12.91 μm in the specimen collected from Konya while that of the other specimen was measured as 9.06 μm.

Section: *Foenum-graecum*

Trigonella gladiata Stev. Fischer, 1808, 2n=16 (Fig. 1T). All chromosome pairs at the somatic metaphase phase were observed to be metacentric. The chromosome length ranges between 2.03 and 4.72 μm. The total haploid chromosome length was measured as 27.70 μm. *T. cariensis* Boiss. In the specimen collected from İzmir province; 2n=16 (Fig. 1, U), and in the specimen collected from Antalya province; 2n=16 (Fig. 1, V). Both specimens have four pairs of metacentric and four pairs of submetacentric chromosomes at somatic metaphase. Of the two specimens, the former has chromosomes whose lengths vary from 2.66 to 3.94 μm with the total haploid chromosome length of 25.28 μm. For the latter, the chromosome length ranges between 3.93 and 6.28 μm, and the total haploid chromosome length was measured as 39.78 μm. In addition, one pair of satellite metaphase chromosomes has been detected in the specimen from Antalya province. *T. foenum-graecum* L., 1753, 2n=16 (Fig. 1, W), and *T. macrorrhyncha* Boiss., 1843, 2n=16 (Fig. 1, X). At somatic metaphase, two pairs of metacentric and six pairs of submetacentric chromosomes were observed for both species. For *T. foenum-graecum*, the chromosome length ranges between 3.03 and 4.84 μm, and the total haploid chromosome length was measured as 30.23 μm. Endemic to Turkey, *T. macrorrhyncha* has chromosomes varying from 2.23 to 4.40 μm. In this species, the total haploid chromosome length is 25.67 μm. Idiograms of each species were arranged in order of decreasing length (Fig. 2). The total haploid chromosome lengths were given in Table 2, and the information of the 19 species studied were presented in Table 3.

Discussion

1. Basic chromosome number variations

In this karyological study, two different basic chromosome numbers of x=7 and x=8 were observed in the species belonging to ten sections of *Trigonella*. Bidak and Amin (1996) reported two different basic chromosome numbers of x=8 and x=9 for *Trigonella* species studied. Among studied species, only two species, *T. plicata* and *T. rostrata*, from the sections of *Pectinatae* and *Lunatae*, respectively, have the basic chromosome number of x=7.

2. Chromosome number and morphology variations

Two different somatic chromosome numbers (2n=14 and 2n=16) were observed in studied sections. The smallest chromosome length is 0.43 μm measured in *T. cephalotes* (section *Uncinatae*) while the biggest of that is 6.28 μm measured in *T. cariensis* (section *Foenum-graecum*). The smallest total haploid chromosome length was measured as 4.49 μm from the species of *T. cephalotes* (section *Uncinatae*). *T. cariensis* (section *Foenum-graecum*) has the biggest total haploid chromosome length of 39.78 μm. *T. rostrata* (section *Lunatae*) has the smallest arm ratio (1.17), and *T. foenum-graecum* (section *Foenum-graecum*) has the biggest (2.17). The smallest centromeric index (4.18) was measured in *T. foenum-graecum* (section *Foenum-graecum*) while the biggest of that (6.56) was observed in *T. rostrata* (section *Lunatae*). In this study, there is a marked difference in somatic chromosome lengths compared to other species of the section *Foenum-graecum*. Chromosome numbers are rather close to each other excluding several species (*Lunatae*, *T. corniculata* and *T. spinosa*) in other sections. Besides, 2B chromosome was observed in *T. procumbens* in the section *Capitatae*.

Karyotype formulae of the sections of *Samaroideae*, *Pectinatae*, *Lunatae*, *Isthmocarpae*, *Capitatae* and *Biebersteinianae* are completely composed of metacentric chromosome pairs. Karyotype formulae of species in other sections are composed of metacentric and submetacentric chromosome pairs. While there is one pair of satellite metaphase chromosomes in *T. lunata* (section *Lunatae*), *T.cariensis* (section *Foenum-graecum*) has two pairs of satellite metaphase chromosomes. Section *Samaroideae* is represented by a single species (*T. cretica*) in Turkey. The karyotype of this species has been analysed by Yılmaz (2006) reporting the karyotype formulae 2n=16 as in our study. Our results agree with one of the reports of somatic chromosome number of 2n=16 from the same locality (Yılmaz 2006).

Section *Pectinatae* is represented by a single species (*T. plicata*) in Turkey. Karyotype analysis of this species has been performed by us for the first time. Section *Luna-*

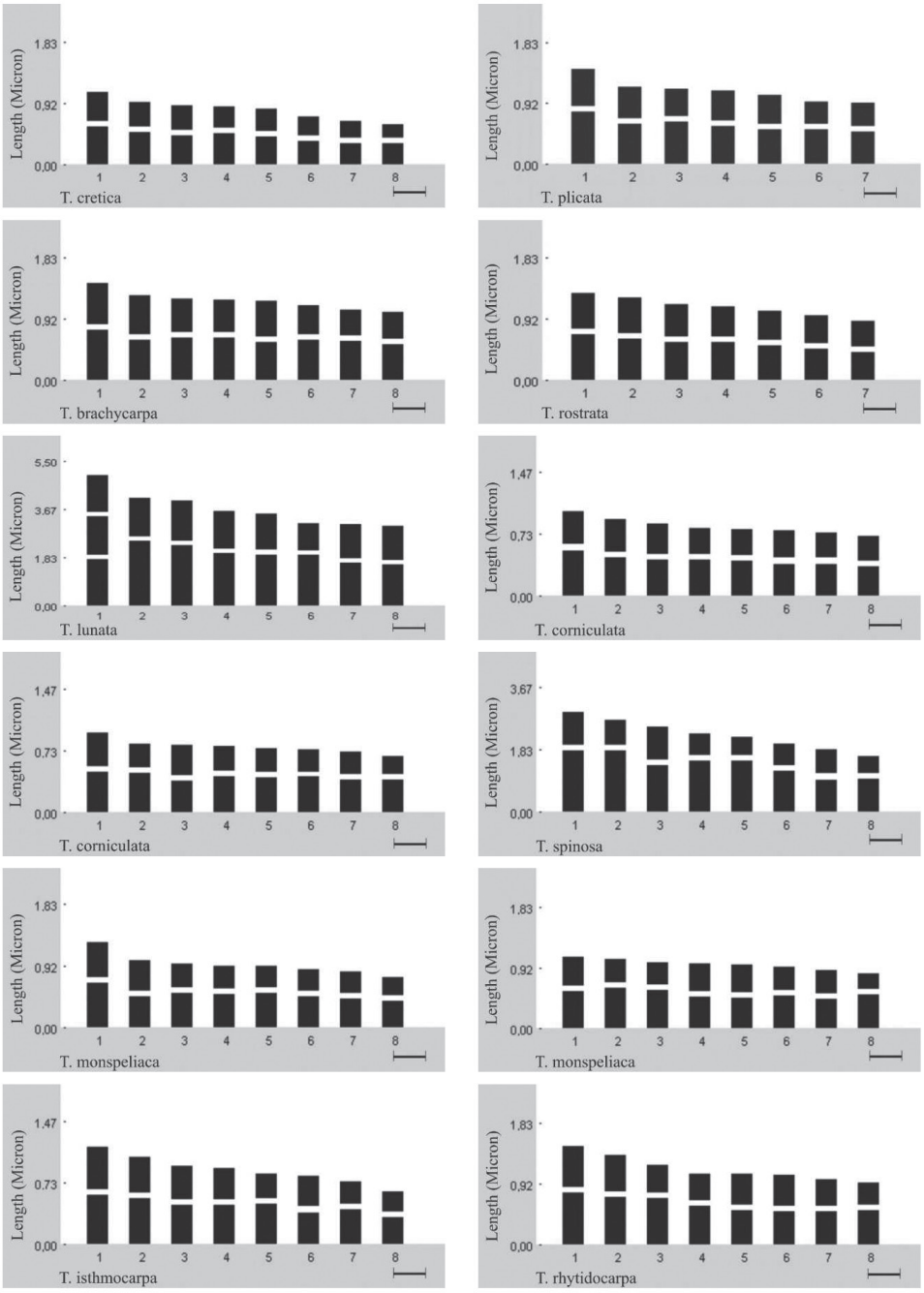

Figure 2a. Idiograms of *Trigonella* species (*T. corniculata* no: 4616 and 3391; *T. monspeliaca* no: 3358 and no: 3327; (*T. coerulescens* no: 3587and 3659; *T. cariensis* no: 3332 and 4620).

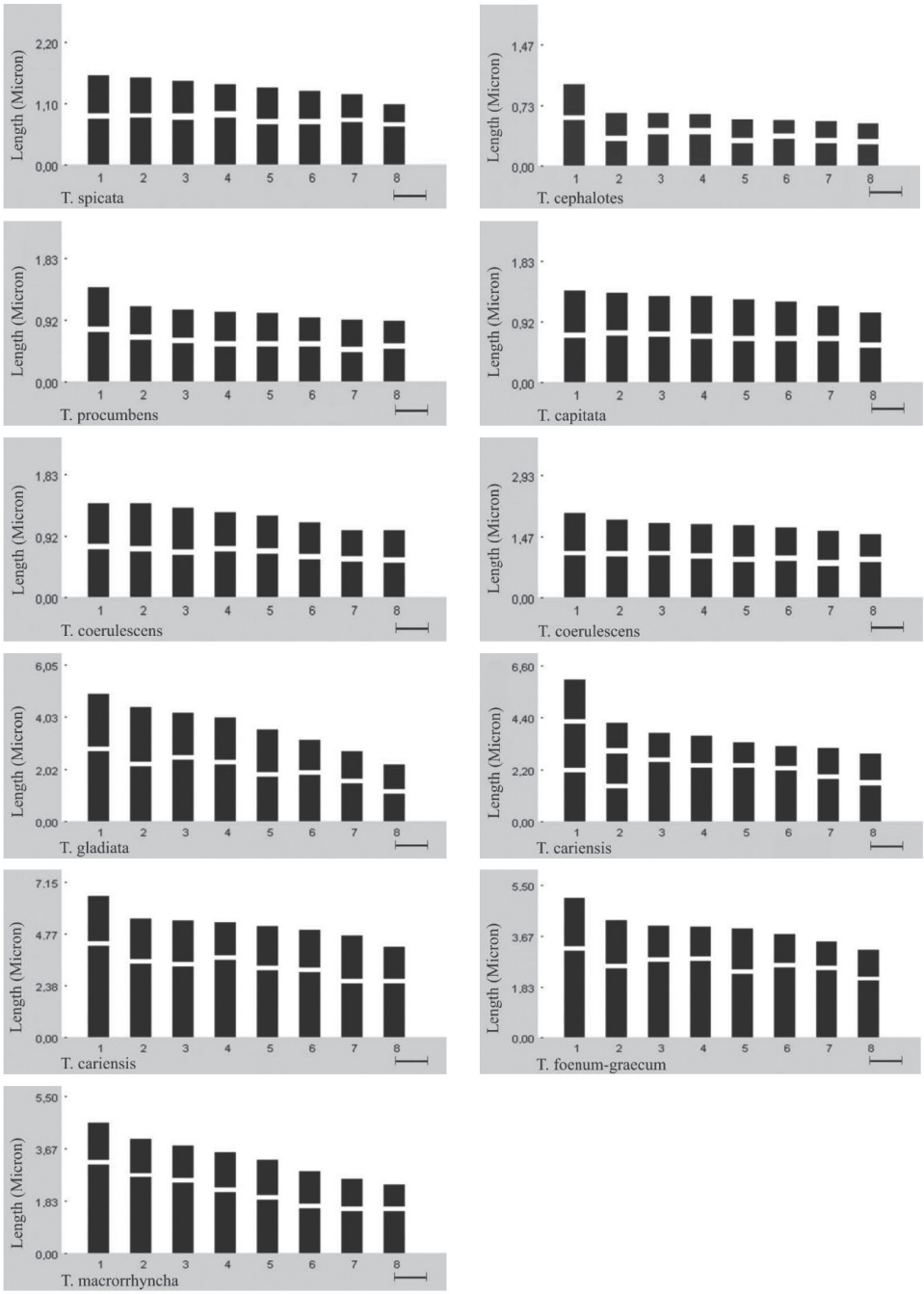

Figure 2b. Idiograms of *Trigonella* species (*T. corniculata* no: 4616 and 3391; *T. monspeliaca* no: 3358 and no: 3327; (*T. coerulescens* no: 3587and 3659; *T. cariensis* no: 3332 and 4620).

Table 2. Chromosome comparison in the examined species of *Trigonella* (AR: arm ratio; CI: centromeric index; THC: total length of haploid complement; M: metacentric; SM: submetacentric; *one pair of satellites is shown in the chromosome pairs are marked with an asterisk).

Sections and species	Chromosome numbers (2n=2x)	Chromosome sizes (µm)	AR	CI	THL (µm)	M	SM
Section *Samaroideae*	16	0.52–1.01	1.33	5.40	5.88	8	-
T. cretica							
Section *Pectinatae*	14	0.84–1.34	1.37	6.06	7.12	7	-
T. plicata							
Section *Lunatae*	16	0.94–1.36	1.26	5.58	8.85	8	-
T. brachycarpa	14	0.81–1.22	1.17	6.56	7.07	7	-
T. rostrata	16	2.85–3.92	1.46	5.12	26.44	8	-
*T. lunata**							
Section *Falcatulae*	16	0.61–0.88	1.46	5.15	5.71	7	1
T. corniculata	16	1.54–2.57	1.78	4.59	17.58	4	4
T. corniculata	16	1.49–2.76	1.99	4.25	16.76	2	6
T. spinosa							
Section *Reflexae*	16	0.66–1.18	1.40	5.26	6.81	8	-
T. monspeliaca	16	0.74–1.00	1.50	5.11	7.06	5	3
T. monspeliaca							
Section *Isthmocarpae*	16	0.57–1.10	1.31	5.47	6.53	8	-
T. isthmocarpa	16	0.85–1.40	1.34	5.37	8.47	8	-
T. rhytidocarpa							
Section *Uncinatae*	16	1.00–1.50	1.52	5.07	10.36	5	3
T. spicata	16	0.43–0.94	1.59	4.96	4.49	5	3
T.cephalotes							
Section *Capitatae*	16+2B	0.82–1.32	1.31	5.43	7.75	8	-
T. procumbens	16	0.96–1.30	1.19	5.68	9.33	8	-
T. capitata							
Section *Biebersteinianae*	16	1.38–1.91	1.32	5.45	12.9	8	-
T. coerulescens	16	0.91–1.32	1.25	5.62	19.06	8	-
T. coerulescens							
Section *Foenum-*	16	2.03–4.72	1.29	5.50	27.70	8	-
graecum	16	2.66–3.94	1.83	4.59	25.28	4	4
T. gladiata	16	3.93–6.28	1.78	4.53	39.78	4	4
*T. cariensis**	16	3.03–4.84	2.17	4.18	30.23	2	6
T. cariensis	16	2.23–4.40	1.85	4.37	25.67	3	5
T. foenum-graecum							
T. macrorrhyncha							

tae is represented by four species (*T. brachycarpa*, *T. rostrata*, *T. lunata* and *T. sirjaevii* Hub.-Mor., 1939) in Turkey. Making a general evaluation of the section, diploid chromosome numbers were found to be different although the localities of the two types (*T. brachycarpa* and *T. rostrata*) in the section were similar. The diploid chromosome number is 2n=16 in *T. brachycarpa* whereas it is 2n=14 in *T. rostrata*. The smallest chromosome length of 0.81 µm was measured in *T. rostrata* in this section while the biggest

Table 3. The information of the 19 species studied.

Section	Species	Chromosome number (2n)	Chromosome numbers reported (2n)	References
Samaroideae	*T. cretica*	16	16	Yılmaz, 2006
Pectinatae	*T. plicata*	14	-	-
Lunatae	*T. brachycarpa*	16	-	-
"	*T. rostrata*	14	-	-
"	*T. lunata*	16	-	-
Falcatulae	*T. corniculata*	16	16	Tutin, Heywood, 1964
"	*T. spinosa*	16	16	Bidak, Amin, 1996
Reflexae	*T. monspeliaca*	16	16	Darlington, Wylie, 1955
Isthmocarpae	*T. isthmocarpa*	16	-	-
"	*T. rhytidocarpa*	16	-	-
Uncinatae	*T. spicata*	16	-	-
"	*T. cephalotes*	16	-	-
Capitatae	*T. procumbens*	16 + 2B	18	Yılmaz, 2006
"	*T. capitata*	16	-	-
Biebersteinianae	*T. coerulescens*	16	16	Yılmaz, 2006
Foenum-graecum	*T. gladiata*	16	16	Bidak, Amin 1996 Darlington, Wylie, 1955 Ladizinsky, Vosa, 1986
"	*T. cariensis*	16	16	Ladizinsky, Vosa, 1986
"	*T. foenum-graecum*	16	16	Ladizinsky, Vosa 1986; Bal, 1990; Tutin, Heywood, 1964
"	*T. macrorrhyncha*	16	16	Ladizinsky, Vosa, 1986

of that was observed in *T. lunata* with a length of 3.92 µm. *T. rostrata* is the species with the smallest arm length of 1.17; however, it is also the species with the biggest centromeric index (6.56). *T. lunata* is the one with the biggest total haploid chromosome length of 26.44 µm. This length is rather different compared to the species in other sections. Besides, one pair of satellite chromosome has been observed in *T. lunata*. Karyotype formulae of all species in the section are composed of metacentric chromosome pairs. *T. sırjaevii* could not been studied due to its inability to be germinated.

Section *Falcatulae* is represented by two species (*T. corniculata* and *T. spinosa*) in Turkey. *T. corniculata* was studied in two different localities (İzmir and Muğla). In Muğla case, tetraploidy was observed differing from the other. Total haploid chromosome length was measured as 5.71 µm in İzmir province while that was 17.58 µm in Muğla province. It can be stated that the distinction between the karyological values obtained from these two localities resulted from locality differences. In Izmir province, the karyotype formula was 7m+1sm whereas it was set as 4m+4sm in Muğla province. Our diploid counts are in agreement with the literature such as one of the reports

from Turkish material and many others from different territories (Tutin and Heywood 1964). From a karyological point of view, to obtain same results from the same species confirms the previous studies. The species of *T. spinosa* is also placed in the section *Falcatulae*. Compared with the other species (*T. corniculata*) of the section, the chromosome number is the same and the chromosome size measured is very close to each other. However, karyotype formulae are different. Karyotype formulae of *T. spinosa* is 2m+6sm. Bidak et Amin (1996) reported the somatic chromosome numbers as 2n=16 and 18 in *T. gladiata*, 2n=16 in *T. ornithopodiodes*, 2n=16 in *T. spinosa* and 2n=18 in *T. stellata*.

The section *Reflexae* is represented by a single species (*T. monspeliaca*) in Turkey. This species was studied in two different localities. Diploid chromosome numbers are the same (2n=16) in both localities of *T. monspeliaca* species. The chromosome sizes, total haploid chromosome lengths, arm lengths and centromeric indices are very close to each other while the karyotype formulae are different, i.e. the karyotype formula of Muğla province is 8m, of Antalya province is 5m+3sm. For Darlington and Wylie (1955), in a cytological study conducted on species belonging to *Trigonella*, diploid chromosome numbers varied from 2n=16 to 2n=32. For example, they are 2n=16 in *T. gladiata*, 2n=16 in *T. monspeliaca*, 2n=28, 30 and 2n=32 in *T. polyceratia*.

The section *Isthmocarpae* is represented by two species (*T. isthmocarpa* and *T. rhytidocarpa*) in Turkey. Karyology of the two species in this section was studied for the first time. The diploid chromosome number of *T. isthmocarpa* and *T. rhytidocarpa* species were found to be 2n=16=8m. Karyological characteristics of these two species are very close to each other.

The section *Uncinatae* is represented by two species (*T. spicata* and *T. cephalotes*) in Turkey. The diploid chromosome number of *T. spicata* and *T. cephalotes* were found to be 2n=16=5m+3sm. Karyological characteristics of these two species are very close to each other. There is a marked difference only in terms of total haploid chromosome length. While total haploid chromosome length was 10.36 μm in *T. spicata*, that is 4.49 μm in *T. cephalotes*. *T. cephalotes* is also the species having the smallest haploid chromosome length among studied species.

The section *Capitatae* is represented by three species (*T. procumbens*, *T. capitata* and *T. pseudocapitata*) in Turkey. The diploid chromosome number of *T. procumbens* and *T. capitata* was found as 2n=16=8m. Karyological characteristics of these two species are very close to each other. In addition to A chromosomes, two examples of B chromosomes were observed in *T. procumbens* differing from the other species of the sections studied. The chromosome number of *T. procumbens* was reported as 2n=18 by Yılmaz (2006) from the same locality, but he did not mention B chromosomes. The other species of the section, *T. pseudocapitata*, could not be studied due to its inability to germination.

The section *Biebersteinianae* is represented by *T. coerulescens* in Turkey. It was studied from two different localities. Diploid chromosome numbers are the same (2n=16=8m) in both localities of *T. coerulescens*. Chromosome sizes, total haploid chromosome lengths, arm lengths and centromeric indices are very close to each other.

The chromosome number of *Trigonella coerulescens* is in agreement with the previous report (2n=16) by Yılmaz (2006).

The section *Foenum-graecum* is represented by five species (*T. gladiata*, *T. cariensis*, *T. foenum-graecum*, *T. macrorrhyncha* and *T. cassia*) in Turkey. Diploid chromosome numbers of the four species studied in this section are the same (2n=16). *T. cassia* could not be studied due to failure to germinate. The chromosome morphologies of the species are very close to each other. The karyotype formula of *Trigonella gladiata* is 8m. In a cytological study performed by Bidak & Amin (1996), diploid chromosome number was found to be 2n=16 and 2n=18 in *T. gladiata*. Researchers reported two different basic chromosome numbers for this species (x=8 and x=9). However, the basic chromosome number in our study was found to be x=8 for this species. In a cytological study conducted on *Trigonella* species, Darlington and Wylie (1955) reported that the diploid chromosome numbers varied from 2n=16 to 2n=32. For example, 2n=16 in *T. gladiata*, 2n=16 in *T. monspeliaca*, 2n=28, 30 and 32 in *T. polyceratia*. Somatic chromosome numbers found in our study are parallel with that report. Two double satellite chromosome pairs were observed in a sample collected from Antalya. The sample collected from İzmir has the biggest total haploid chromosome length (39.78 μm) among all sections. has diploid chromosome number of 2n=16 as in other species in the section. The karyotype formula is 2m+6sm. *T. foenum-graecum* is the species with the biggest arm ratio in all sections (2.17). Chromosome numbers of only two *Trigonella* species were reported in European Flora records, *T. corniculata* 2n=16 and *T. foenum-graecum* 2n=16 (Tutin and Heywood 1964). Our results obtained from this study agree with that report. In a study on karyotype analysis of *T. foeanum-graecum*, somatic chromosome numbers were found to be similar to that of our study (Bal, 1990). The diploid chromosome number of *T. macrorrhyncha* is 2n=16 as in all other species in the section. The karyotype formula is 3m+5sm. In another study conducted in six different species (*T. gladiata*, *T. cariensis*, *T. foenum-graecum*, *T. berythea*, *T. macrorrhyncha* and *T. cassia*) of the section *Foenum-graecum*, diploid chromosome numbers were reported as 2n=16 (Ladizinsky and Vosa, 1986). In this case, our counts agree with the previous study. It is considered that the results obtained from this karyological study have contributed to the taxonomical revision of the genus *Trigonella*.

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Ommexecha virens (Thunberg, 1824) and *Descampsacris serrulatum* (Serville, 1831) (Orthoptera, Ommexechidae): karyotypes, constitutive heterochromatin and nucleolar organizing regions

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Abstract

Chromosomes of *Ommexecha virens* and *Descampsacris serrulatum* (Ommexechidae) were analyzed through conventional staining, C-banding, base specific fluorochromes, silver nitrate impregnation ($AgNO_3$), and fluorescent in situ hybridization (FISH) with probe for 45S rDNA. The two species presented diploid number 2n= 23,X0 in males and acrocentric autosomes, except the pair one that presented submetacentric morphology. The X chromosome has distinct morphology in the two analyzed species, being a medium acrocentric in *O. virens* and large submetacentric in *D. serrulatum*. The C-banding revealed pericentromeric blocks of constitutive heterochromatin (CH) in all the chromosomes of *D. serrulatum*. For *O. virens* it was evidenced that the blocks of CH are preferentially located in the pericentromeric area (however some bivalents presents additional blocks) or in different positions. The staining with CMA_3/ DA/DAPI showed GC rich CH blocks (CMA_3^+) in some chromosomes of the two species. The nucleolar organizer regions (NORs) were located in the bivalents L2, S9, S10 of *O. virens* and M5, M6, M7, S11 of *D. serrulatum*. The FISH for rDNA showed coincident results with the pattern of active NORs revealed by $AgNO_3$. This work presents the first chromosomal data, obtained through differential cytogenetics techniques in Ommexechidae, contributing to a better characterization of karyotypic evolution for this grasshopper family.

Keywords
FISH, grasshopper, Ommexechidae**,** rDNA, *Ommexecha virens, Descampsacris serrulatum*

Introduction

The family Ommexechidae is endemic to South America, constituted by two subfamilies (Aucacrinae and Ommexechinae) and includes about 12 genera and 30 species. *Descampsacris* is a monotypic genus represented by *D. serrulatum*. On the other hand, the genus *Ommexecha* comprises seven species, *Ommexecha apolinari, O. brunneri, O. germari, O. giglio-tosi, O. gracilis, O. macropterum* and *O. virens*. This genus has a wide geographical distribution being found from the Andes to Caribbean. Among the described species, *O. virens* presents great morphologic and chromatic variability (Ronderos, 1979).

Mesa et al. (1982) reported cytogenetic data on 13 Ommexechidae species, mainly with karyotype 2n= 23, X0. However, *Ommexecha germari* has the smallest chromosome number (2n= 21, X0) and *Conometopus sulcaticollis* showed 2n= 25, X0, the largest diploid number for the family. *Spathalium helios* demonstrates 2n = 22, neoXY with the X chromosome metacentric corresponding to the largest element of the karyotype and Y showed acrocentric morphology (Mesa et al. 1990). This species demonstrates also the unique representative of Ommexechidae analyzed by differential staining technique, showing constitutive hetrochromatin (CH) in the centromeric region of the autosomes and additional terminal blocks in the pairs seven and eight (Mesa et al. 1990).

Differential cytogenetic staining in grasshoppers of the families Acrididae and Romaleidae, from Neotropical Region, has shown a variability in distribution pattern and qualification of the CH through the C-banding and base specific fluorochromes Chromomycin A_3 (CMA_3) and 4'-6'-diamidino-2-fellindol (DAPI) staining. Distinct nucleolus organizing regions (NORs) locations, through the silver nitrate impregnation ($AgNO_3$) and fluorescent *in situ* hybridization (FISH), have been observed in these species (Souza et al. 1998, Loreto and Souza 2000, Pereira and Souza 2000, Souza et al. 2003, Loreto et al. 2005, Souza and Melo 2007, Loreto et al. 2008).

In this work chromosomes of *Ommexecha virens* and *Descampsacris serrulatum* were analyzed through conventional staining, C-banding, base specific fluorochromes, impregnation with $AgNO_3$ and FISH with probe of 45S rDNA. The karyotypic patterns obtained contributed to a better understanding about chromosomal evolution in the family Ommexechidae.

Material and methods

In this work 36 males of *O. virens* (Thunberg, 1824) and 11 of *D. serrulatum* (Serville, 1831) were analyzed. The species studied were collected in different areas in the states of Pernambuco and Bahia in the Northeast Region of Brazil (Table 1). The testes were fixed in ethanol and acetic acid 3:1. The cytological preparations were obtained through squashing of testes follicles. For conventional analysis the slides were stained with lacto acetic orcein 2%. For the C-banding the technique of Sumner (1972) was used with small modifications in the time of use of the basic solution. In the sequence the slides were treated with HCl 0,2N by 25 minutes, barium hydroxide ($Ba(OH)_2$) to 5% the (60°C) for 10 seconds and 2XSSC the (60°C) for 25 minutes. The slides were stained with 5% Giemsa during 5 minutes. The staining of CMA_3/DA/DAPI was accomplished in agreement with Schweizer et al. (1983). After 5 days the slides were stained with CMA_3 during 50 minutes, washed in distilled water and stained with Distamycin during 45 minutes, again washed and stained with DAPI for 20 minutes. The slides were mounted in glycerol/ Macllvaine buffer/$MgCl_2$.

The silver nitrate impregnation ($AgNO_3$) was done according to Howell and Black (1980). A 50% solution of silver nitrate was used (5g of $AgNO_3$ in 10ml of distilled water), besides a colloidal solution (gelatin). The slides were incubated in humid camera at 70ºC during from 3 to 5 minutes.

Fluorescent *in situ* hybridization (FISH) was performed according to Moscone et al. (1996) using probe for 45S rDNA. The probe was labelled with digoxigenin through nick translation and detected with the anti-digoxigenin sheep antibody linked to the fluorochrome FITC. A FITC-conjugated rabbit anti-sheep antibody (743, Dako) was used for amplification of hybridization signal. The chromosomal preparations were counterstained with DAPI (2µg/ml) and mounted in Vectashield H-1000 (Vector).

The cells submitted to the fluorochromes and FISH were captured through the image capture system Cytovision coupled to the microscope Olympus BX51. For the other methods the cells were photographed in microscope Leica, using a film Kodak Imagelink, Wing 25. The figures were mounted with the use of the program Corel Draw Graphics Suite 12 software.

Results

Descampsacris serrulatum and *Ommexecha virens* demonstrate similar karyotypes with diploid number 2n= 23,X0 in the males and acrocentric autosomes, except the pair one that presented submetacentric morphology (Fig. 1, a-f). The autosomes were arranged according to their size as three large (L1-L3), five medium (M4-M8) and three small (S9-S11). *D. serrulatum* has one large X chromosome with submetacentric morphology (Fig. 1, a, b), while in *O. virens* the X is of medium size and acrocentric (Fig. 1, c, d). During the prophase I of the two species the X chromosome was heterop-

Table 1. Species, localities of collections, geographical coordinates and number of analyzed individuals.

Species	Localities	Coordinates	No. of analyzed individuals
Ommexecha virens (Serville, 1831)	Buíque (PE)	8°37'23"S; 37°9'12"W	8
	Itamaracá (PE)	7°44'52"S; 34°51'19"W	9
	Sobradinho (BA)	9°27'19"S; 40°49'24"W	6
	Rio de Contas (BA)	13°34'44"S; 41°48'41"W	7
	Itaberaba (BA)	12°31'39"S; 40°18'25"W	6
Descampsacris serrulatum (Thunberg, 1824)	Rio de Contas (BA)	13°34'44"S; 41°48'41"W	6
	Andaraí (BA)	12°48'26"S; 41°19'53"W	3
	Mucugê (BA)	13°0'19"S; 41°22'45"W	2

ycnotic positive until the diplotene, being that behavior reverted in the metaphase I (Fig. 1, b-d).

C-banding revealed distribution of constitutive heterochromatin (CH) in the pericentromeric region of all chromosomes of *D. serrulatum* (Fig. 2, b). In *O. virens* has blocks of CH preferentiality in the pericentromeric region, however some bivalents demonstrate additional blocks in different positions, such as L2 with proximal block, besides the pericentromeric; M8 showed intercalation of eu-heterochromatin along its extension; S9 presented a large block of CH extending from the pericentromeric to proximal region. Moreover the X chromosome showed proximal block (Fig. 2, a).

CMA_3/DA/DAPI staining revealed blocks CMA_3^+ in some chromosomes of the karyotype of *O. virens*, as well as of *D. serrulatum* (Fig. 2, c, e). However, no AT positive blocks were detected in these two species (Fig. 2, d, f). Impregnation with $AgNO_3$ identified nucleolus organizing regions (NORs) active in the two analyzed species. In *D. serrulatum*, NORs are located in four autosomal bivalents (M5, M6, M7 and S11) and in *O. virens* in three (L2, S9 and S10) (Fig. 3, a, c).

FISH with probe of 45S rDNA was used in meiotic cells of *D. serrulatum* and *O. virens* allowing the precise identification of rDNA sites observed by the impregnation $AgNO_3$. In *D. serrulatum* it was observed that in four autosomal pairs the sites of rDNA are located in the pericentromeric regions (Fig. 3, b), besides the bivalent S11 presented a large sign of the hybridization, occupying about 2/3 of the chromosome length. In *O. virens* it was revealed that the bivalents L2 and S9 possess pericentromeric sites and the S10 proximal (Fig. 3, d).

Discussion

In spite of the wide distribution of the Ommexechidae in South America, the cytogenetic studies in this family are scarce and based mainly on conventional method (Mesa et al. 1982). In the present study conventional chromosomal analysis showed that the two species analyzed have 2n= 23, X0 in the males with a remarkable large

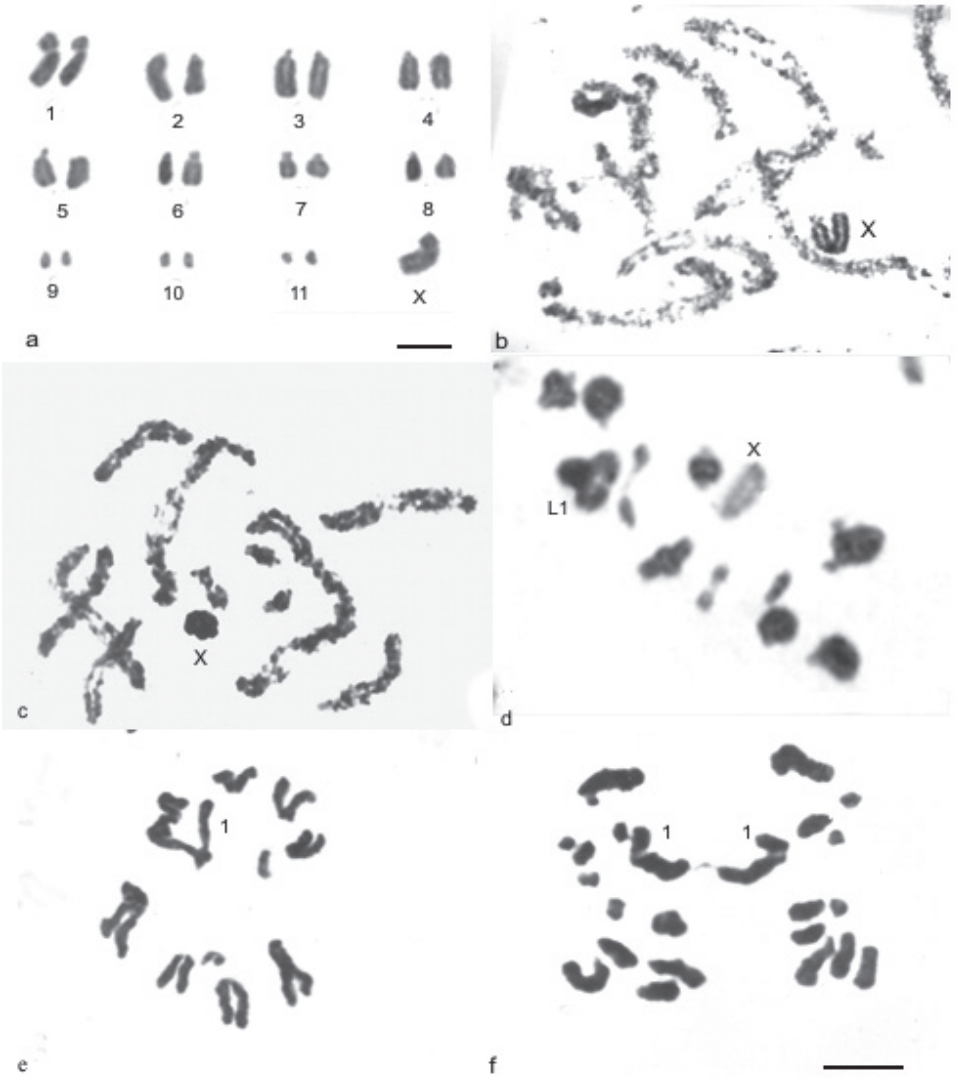

Figure 1. Conventional staining in mitotic and meiotic cells of *Descampsacris serrulatum* **a, b** and *Ommexecha virens* **c, f**. **a** spermatogonial metaphase **b** and **c** pachytene **d** metaphase I **e** metaphase II **f** anaphase II. Bar= 5μm.

submetacentric bivalent (pair one). The occurence of a large autosomal submetacentric pair were frequently reported for Ommexechidae. This submetacentric pair probably originated from a pericentric inversion involving an original acrocentric chromosome of a common ancestor of the discussed species, representing a karyotypic marker for this family, occurring in 16 of the 19 species studied until now (Mesa and Ferreira 1977, Mesa et al. 1982, 1990). On the other hand *Clarazella bimaculata, Conometopus sulcaticollis* and *Pachyossa signata* demonstrate only acro-

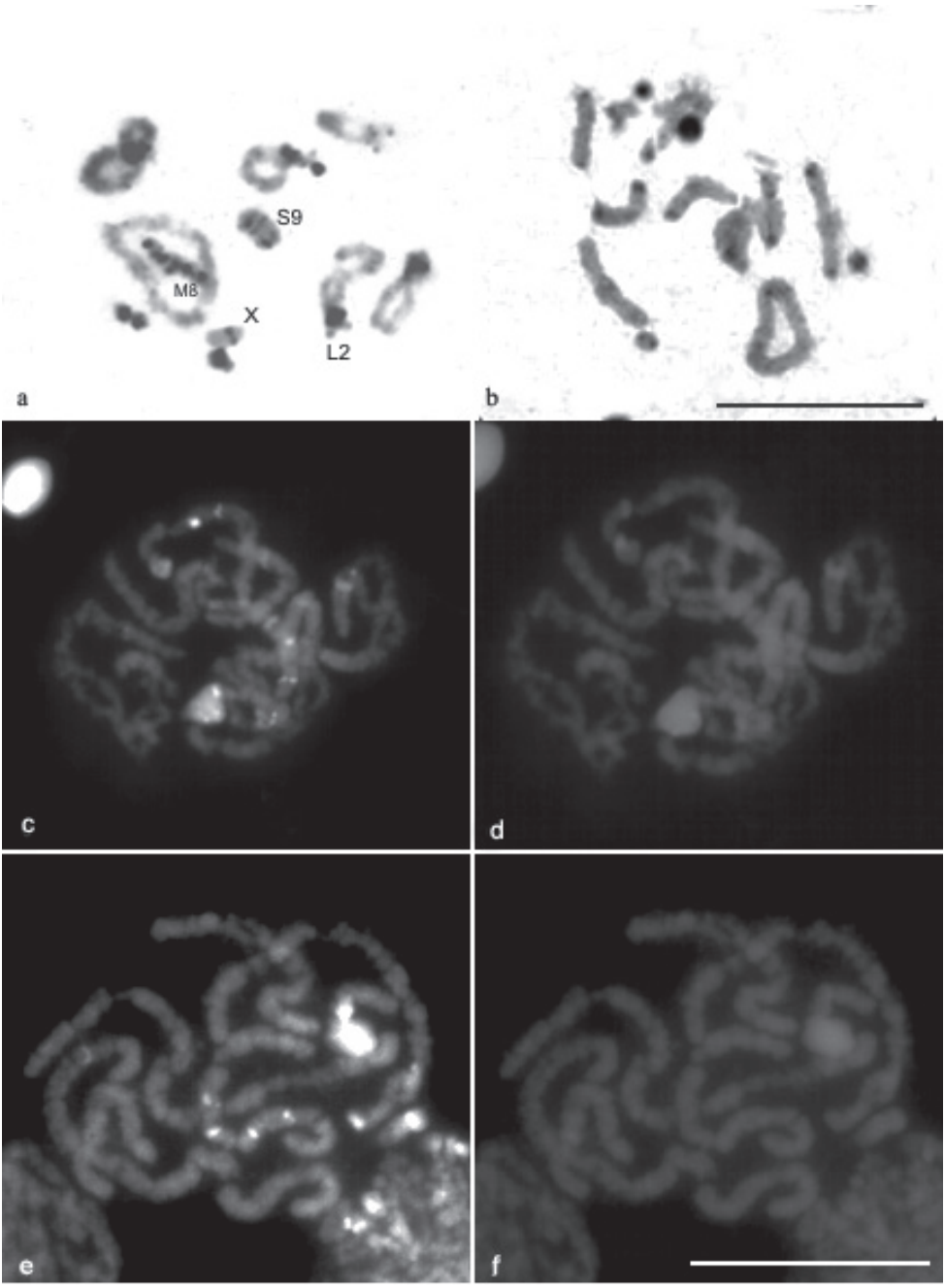

Figure 2. C-banding pattern in diplotene cells of *Ommexecha virens* **a** and *Descampsacris serrulatum* **b** Staining CMA_3/DA/DAPI in pachytene cells of *Descampsacris serrulatum* **c** CMA_3 **d** DAPI and *Ommexecha virens* **e** CMA_3 **f** DAPI. Bar= 5 μm.

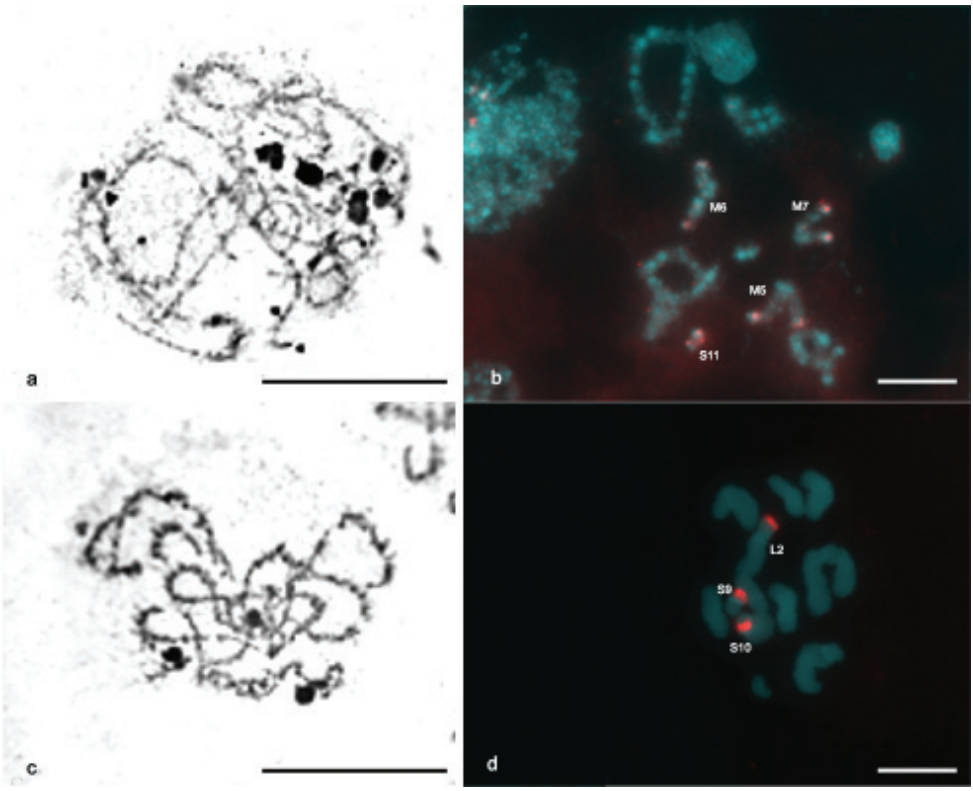

Figure 3. Impregnation with silver nitrate **a, c** and FISH with probe of 45S rDNA **b, d**. Zygotene and diakinesis of *Descampsacris serrulatum* **a, b** and zygotene and pachytene of *Ommexecha virens* **c, d**. In **b, d** are indicated the pairs containing the rDNA sites. Bar= 5 μm.

centric chromosomes in their karyotype. However, *D. serrulatum* and *O. vir*ens have autosomes with the same morphology. The first species shows the X chromosome with submetacentric morphology, while the second one has acrocentric X chromosome. The difference in the morphology of the X chromosome of the two species probably is a result of pericentric inversion from the ancestral acrocentric condition. In general, the karyotypes of *O. virens* and *D. serrulatum* coincide with karyotypes of most other cytogenetically studied species of Ommexechidae and with data of Mesa et al. (1982) although the X submetacentric of *D. serrulatum* has not been observed by these authors.

The pericentromeric pattern of distribution of constitutive heterochromatin observed in *D. serrulatum* and *O. virens* is quite common for the superfamíly Acridoidea. This pattern has also been described for several species of the Neotropical Region, belonging the families Acrididae and Romaleidae (Souza and Kido 1995, Rocha et al. 1997, Loreto and Souza 2000, Rocha et al. 2004, Loreto et al. 2005,

Souza and Melo 2007). In Ommexechidae the only species, *Spathalium helios*, was studied with chromosomal C-banding technique until now. This species has pericentromeric blocks of CH, besides telomeric blocks in the pairs 7 and 8 (Mesa et al. 1990). For the two species analyzed in this work, significant differences were observed in some chromosomal pairs of *O. virens* (L2, M8, S9 and X) with CH in different positions or in larger amount than it was observed for the same chromosomal pairs of *D. serrulatum*. This difference can be attributed to amplification mechanisms or heterochromatin dispersion that are acting more intensely in *O. virens* than in *D. serrulatum*.

The pattern of qualification of CH visualized by the staining CMA_3/DA/DAPI in *D. serrulatum* and *O. virens* showed blocks of positive CMA_3 in some chromosomes of the karyotype. Similar patterns with the presence of CMA_3^+ blocks in some chromosomes were also described in *Belosacris coccineipes, Cornops aquaticum, Stenopola dorsalis, Stenacris xantochlorae* and *Tucayaca parvula* (Acrididae) (Loreto and Souza, 2000, Rocha et al. 2004). In *Chromacris nuptialis* and *C. speciosa* (Romaleidae) the CMA_3^+ blocks are restrict to one autosomal pair (Loreto et al. 2005). These patterns contrast with the described for *Xyleus angulatus, Phaeoparia megacephala* and *Xestotrachelus robustus* (Romaleidae) in which CH of all karyotypic complement demonstrates the richness for GC base pairs (Souza et al. 1998, Pereira and Souza 2000, Souza et al. 2003).

The references on the using of the $AgNO_3$ impregnation and fluorescent *in situ* hybridization (FISH) for major rDNA show that in grasshoppers as a whole and in species belonging to the families Acrididae and Romaleidae, NORs are more frequently found in one or two autosomal bivalents (Rufas et al. 1985, Souza et al. 2003, Rocha et al. 2004, Souza and Melo 2007, Loreto et al. 2008). Variability in relation to NORs pattern location was observed in two analyzed species. *O. virens* showed active NORs in the pairs L2, S9, S10 and *D. serrulatum* demonstrated NORs involving four of autosomes pairs (M5-M7 and S11). The presence of NORs in four autosome pairs is unusual for grasshoppers, and have been described only in *Eyprepocnemis plorans, Heteracris litoralis* and *Gomphocerus sibiricus* (Rufas et al. 1985). Moreover, differential pattern of distribution of NORs was observed by Loreto et al. (2008) in the species belonging to the genus *Rhammatocerus*. *R. brasilensis* has three autosomal pairs bearing NORs and in contrast, *R. brunneri, R. palustris* and *R. pictus* showed NORs in a single autosomal pair.

The differential pattern of distribution of NORs observed in *D. serrulatum* and *O. virens*, could be explained by a probable amplification and dispersion of sites of rDNA (18S, 28S, 5.8S), leaving of an ancestral condition, in which a single autosomal pair would be NORs bearer. On the other hand, there is no coincident NORs bearing pairs among the two species and the possibility of different origins for the pattern of NORs in Ommexechidae can not be discarded.

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Description of karyotype in *Hypostomus regani* (Ihering, 1905) (Teleostei, Loricariidae) from the Piumhi river in Brazil with comments on karyotype variation found in *Hypostomus*

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Abstract

The paper represents a comparative cytogenetic analysis of three populations of *Hypostomus regani* in Brazil. Two populations belong to the Upper Paraná River Basin and the third one, the karyotype of which is described for the first time, was probably introduced into the São Francisco River Basin through transposition from the Piumhi River. Karyotype features of populations of *H. regani* from the Piracicaba and Tietê River Basins are also discussed. The occurrence of *H. regani* in the São Francisco River Basin is reported for the first time here. The study also revealed distinct differences in the location of the Ag-NORs between the analyzed populations that enable individuals from the Piumhi River, Mogi-Guaçu River and Tietê River to be distinguished from one another. Thus, the data obtained indicate the possibility of geographic variation fixing different karyotypes even in the same basin of origin.

Keywords

cytotaxonomy, karyotype diversification, rDNA

Introduction

The family Loricariidae is the second most numerous among fish, with 716 species distributed among 96 genera (Ferraris 2007). These fish are endemic to the Neotropics, occurring from Costa Rica to Argentina (Reis et al. 2003). This considerable diversity

has resulted in constant identification problems and new species have frequently been described (Pereira and Oyakawa 2003, Cardoso and Silva 2004). Recent studies have revealed that the taxonomy of Loricariidae remains poorly resolved (Armbruster 2004), but six subfamilies are recognized: Loricariinae, Hypoptopomatinae, Hypostominae, Neoplecostominae, Lithogeneinae and Delturinae (Reis et al. 2006). Although Reis et al. (2003) consider Loricariidae to be the largest family of catfish in the world, little is known regarding the constitution and organization of the karyotype in this group, which exhibits a tendency to show quite divergent karyotypes (Artoni and Bertollo 2001).

Hypostomus Lacépède, 1803 is the largest genus of the armored catfish family Loricariidae, with approximately 120 nominal species (Weber 2003). It is also one of the better characterized genera among the loricariids from the cytogenetic standpoint, revealing a variation in diploid number from 2n = 54 in *H. plecostomus* (Linnaeus, 1758) (Muramoto et al. 1968, cited in Artoni and Bertollo 2001) to 2n = 84 in *Hypostomus* sp. 2 (Cereali et al. 2008). Although some trends of the karyotype evolution have been described in *Hypostomus* (Artoni and Bertollo 2001, Kavalco et al. 2005, Alves et al. 2006, Kavalco et al. 2005, Milhomem et al. 2010), especially such features as increase in the diploid number by centric fission (Artoni and Bertollo 1996), the number of karyotyped species is still very small compared to the diversity of species known in this genus.

In the present study, a comparative cytogenetic analysis was carried out on three different populations of *H. regani* (Ihering, 1905). Two populations are from the Upper Paraná River Basin and the other, the karyotype of which is described for the first time, was probably introduced into the São Francisco River Basin through the transposition of the Piumhi River. The aim was to investigate the karyotype in these populations, seeking chromosomal characters potentially important for understanding the taxonomy and biogeography of the species.

Material and methods

Sixteen specimens of *H. regani* were examined (8 males and 8 females), collected from the mouth of the Piumhi River at the São Francisco River in the region of the municipality of Piumhi – MG, Brazil (20°20'31.0" S; 45°59'03.4" W, Alt.: 640 m), (Fig. 1, detail). According to C.H. Zawadzki (personal communication), *Hypostomus regani* is characterized as a species with a body covered by small, round, light-colored and generally well-defined spots. It has a high body and relatively long (narrow) head, large eyes and long dorsal fins, generally with rays reaching the spine of the adipose fin when adpressed. It has plates on the abdomen, except for very young specimens. Although distributed throughout the Paraná-Paraguay Basin, its type locality is the Piracicaba River in the state of São Paulo, Brazil.

The specimens were identified and deposited in the Museu Nacional do Rio de Janeiro (MNRJ 32778; MNRJ 32782; MNRJ 32787). The collection authorization (number 472897) was granted by IBAMA [Brazilian Environmental Protection Agency] and the fishing license (number 091/07) was granted by the Instituto Estadual de Floresta de Minas Gerais, Brazil.

Figure 1. Map of Brazil highlighting the large hydrographic basin of the Paraná-Paraguai system, area of natural distribution of *Hypostomus regain*. Detail: area of the divider of the waters of the Upper Paraná and São Francisco River Basins, altered by the transposition of the Piumhi River (with original drainage to the Grande River in the Upper Paraná Basin) to the Upper São Francisco Basin through an artificial channel that links it to the Sujo River (tributary of the São Francisco River). Star (★) and triangle (▼) indicate sampling sites for the *H. regani* populations studied by Artoni and Bertollo (1996, 2001) and Alves et al. (2006) in the Mogi-Guaçu and Tietê River Basins, respectively; circle (●) indicates the sampling site for the *H. regani* specimens analyzed in the present study in the confluence of the Rio Piumhi with to Rio São Francisco, upper Rio São Francisco basin.

Karyotype data on the *H. regani* populations studied by Artoni and Bertollo (1996), Artoni and Bertollo (2001) from the Mogi-Guaçu River Basin and by Alves et al. (2006) from the Tietê River Basin (Fig. 1) were also accessed (Table 1).

Chromosome preparations were obtained from cells from the anterior portion of the kidney, using *in vivo* treatment with colchicine (Bertollo et al. 1978). Nucleolus

organizer regions (NORs) were detected using silver nitrate (Ag-NORs), based on the method described by Howell and Black (1980). C-positive heterochromatin was analyzed using the C-banding method (Sumner 1972).

Fluorescent *in situ* hybridization (FISH) was employed to locate ribosomal genes in the chromosomes. An 18S rDNA probe from the fish *Prochilodus argenteus* (Agassiz, 1829) (Hatanaka and Galetti Jr. 2004) and a 5S rDNA probe from the fish *Leporinus elongatus* Valenciennes, 1849 (Martins and Galetti Jr. 1999) were used to map the rDNA sites on the chromosomes. Both probes were labeled with 14-dATP biotin by nick translation, following the manufacturer's instructions (Bionick Labeling System – Invitrogen). Amplification and detection of the hybridization signals was carried out using the avidin-FITC and anti-avidin biotin complex system (Sigma). FISH signals were viewed based on the method described by Pinkel et al. (1986) and analyzed under an epifluorescence microscope (Olympus BX51). The images of the chromosomes were captured using the CoolSNAP-Pro software program (Media Cybernetics).

Approximately 30 metaphases from each specimen were analyzed in order to determine the modal diploid number (2n), fundamental number (FN) and karyotype formula. The chromosomes were identified based on the approach described by Levan et al. (1964) and classified in the karyotype as metacentric (m), submetacentric (sm), subtelocentric (st) and acrocentric (a).

Results

All the *Hypostomus regani* specimens analyzed in the present study had 2n = 72 chromosomes with a karyotype formula 8m+16sm+20st+28a. The number of arms was FN = 116 (Table 1, Fig. 2a).

Constitutive heterochromatin was distributed in small blocks (Fig. 2,b). The interstitial region of the long arms of subtelocentric chromosomes pairs 14, 18 and 20 as well as acrocentric pairs 23, 26, 27, 28 and 33 had quite evident blocks. Metacentric chromosome pair 1 had fainter labeling in the interstitial region of the short arm. Chromosome pairs 13, 15 and 36 had heterochromatic blocks in the centromeric re-

Table 1. Chromosomal data of the *Hypostomus regani* populations; from Artoni and Bertollo (2001) (Ref. 1), Alves *et al.* (2006) (Ref. 2) and new data from the population introduced into the São Francisco River Basin (Ref. 3).

Locality	2n/FN	Formula	Ag-NOR	Ref.
Rio Mogi-Guaçu, Rio Mogi-Guaçu basin	72/116	10m+20sm+42st/a	Multiple 1 pair "a" large 1 par "st" small	1
Rio Araquá, Rio Tiete basin	72/116	12m+18sm+26st+16a	Multiple 2 pairs "a" largies	2
Confluence of the Rio Piumhi with to Rio São Francisco, upper Rio São Francisco basin	72/116	8m+16sm+20st+28a	Simple 1 pair "st" large	3

gion. Heterochromatin was located in the telomeric region of the long arm in chromosome pair 31. Nucleolar organizing regions (NORs) labeled by silver nitrate were only evident in the short arm of subtelocentric chromosome pair 15 (Fig. 2,c and 4,a). All cells analyzed exhibited heteromorphism in relation to the size of the Ag-NORs.

Fluorescent *in situ* hybridization confirmed the presence of 18S rDNA coinciding with the Ag-NORs as well as the size heteromorphism of the sites (Figs 3,a-c). The 5s rDNA sites were located in four chromosome pairs: in the terminal region of the short arm of two acrocentric pairs; in the centromeric region of one submetacentric pair; and on another chromosome with no evident homologous labeling (Figs 3,b-d).

Discussion

The Piracicaba River, which is a tributary of the Upper Paraná River, is the type locality of *H. regani*, although the natural distribution of the species is related to the Paraná, Paraguay and Uruguay River Basins (Carvalho and Bockmann 2007). The occurrence of *H. regani* in the São Francisco River Basin is reported for the first time here and is added to the data from the available taxonomic sources (Isbrücker 1980, Montoya-Burgos 2003, Weber 2003, Armbruster 2004, Ferraris 2007). *H. regani* likely invaded the São Francisco River Basin after the transposition of the Piumhi River (Moreira-Filho and Buckup 2005). Although the field and taxonomic data confirm the origin of this species in the Upper Paraná River Basin, the data presented here indicate a divergence in the karyotype macrostructure that involves the possibility of geographic variation. The different cytotypes may have been isolated in allopatry approximately six million years ago (this dating is based on the origin time of the basin of San Francisco river) (Montoya-Burgos 2003). On the other hand, we must also consider the possibility of karyotype diversification occurring within the same hydrographic basin, like the Upper Paraná River Basin.

Karyotype differences in natural fish populations that inhabit the same hydrographic basin have been found, for instance, in the genus *Astyanax* Baird et Girard, 1854. E.g. there are at least three different cytotypes of *A.* prope *fasciatus* (Cuvier, 1819) living in sympatry in the Upper Tibagi River, which is a tributary of the Paraná River (Artoni et al. 2006). According to Artoni et al. (2009), events of geographic vicariance stemming from the history of the South American continent are among the factors to be considered in the karyotype diversification of Neotropical freshwater fish. The authors also consider the evolutionary time for the fixation of chromosome rearrangements and the biology of species. We must stress here that *H. regani* is not a great migrator, as with the majority of Loricariidae, the anatomy of which imposes difficulties on the movement of these fish in overcoming physical barriers, such as waterfalls (Paiva et al. 2005). This imposition favors the formation of more restricted population demes, which may accelerate the formation of new cytotypes within geographically isolated areas through the action of genetic drift and the restriction of gene flow. In *Ancistrus* Kner, 1854, for example, the great karyotypic variability may be related to biological and behavioral characteristics of these

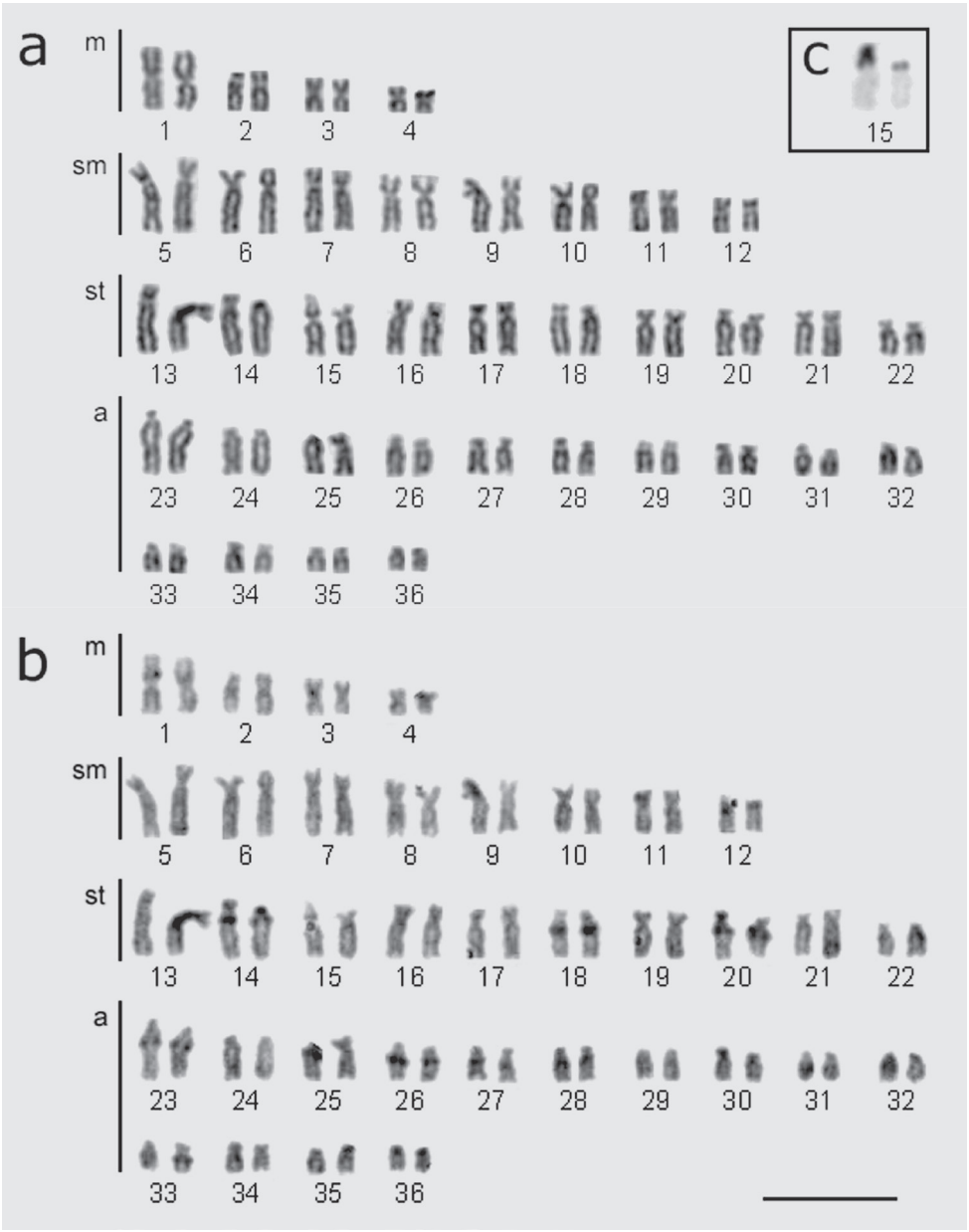

Figure 2. *Hypostomus regani* karyotypes from the confluence of the Piumhi and São Francisco Rivers **a** chromosome stained with Giemsa and **b** sequentially labeled by C-banding; **c** box indicates chromosome pair labeled with silver nitrate locating the nucleolus organizer regions (pair no. 15). Bar = 10 μm.

armored catfish that include microhabitat preferences, territoriality and specialized reproductive tactics, with consequences for the fixation of chromosomal rearrangements and speciation (Oliveira et al. 2009).

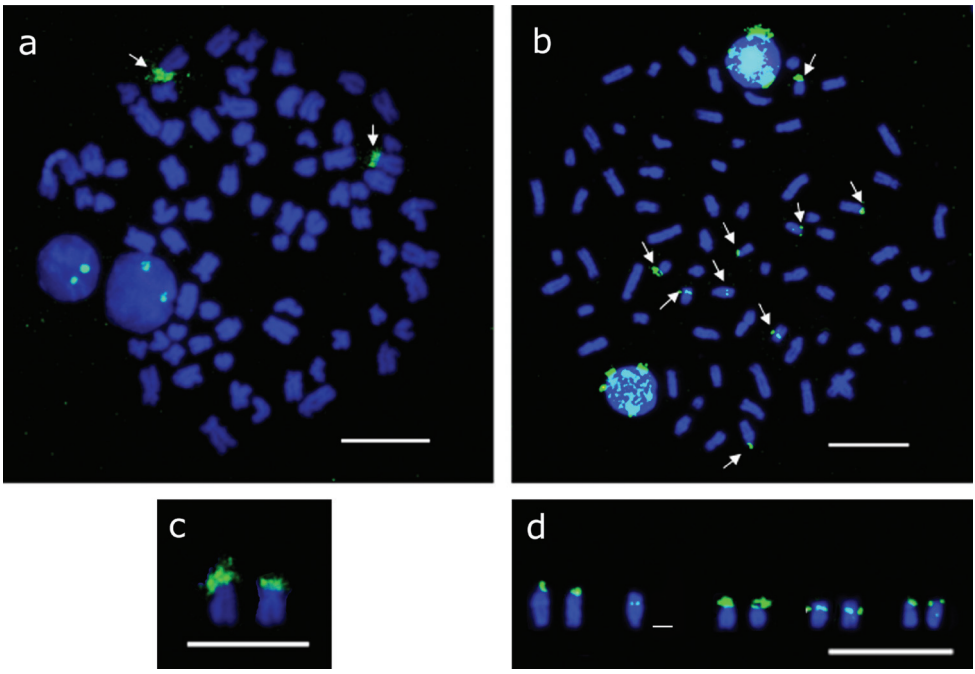

Figure 3. Mitotic metaphases in *Hypostomus regani* from the confluence of the Piumhi and São Francisco Rivers submitted to fluorescent *in situ* hybridization **a** showing two 18S rDNA sites (arrows) **b** nine 5S rDNA sites (arrows) **c** chromosomes bearing 18S rDNA sites **d** chromosomes bearing 5S rDNA sites. Bar = 10 μm.

In a previous study, Artoni and Bertollo (2001) point to evolutionary trends for the karyotype of the subfamilies of Loricariidae. Among those that exhibit extensive variation in the diploid number, the genus *Hypostomus* stands out, with inter-species variation ranging from 2n = 54 to 84 chromosomes, which demonstrates the strong action of events of centric fission in the karyotype diversification of this group when compared to more basal forms found in sister groups of Hypostominae, such as *Liposarcus* Günther, 1864 (2n = 52), *Rhinelepsis* Agassiz, 1829 (2n = 54) and *Pogonopoma* Regan, 1904 (2n=54) (Artoni et al. 1999, Artoni and Bertollo 2001). The data present here for *H. regani*, in relation for the most basal *Hypostomus* species, also support the Artoni and Bertollo's hypothesis (*op. cit.*) regarding the location and distribution of heterochromatin, especially in relation to the accumulation of equilocal and interstitial heterochromatic blocks preferentially located in subtelocentric and acrocentric chromosomes. Artoni and Bertollo (1999) propose that this tendency increases among species of *Hypostomus* that have higher diploid numbers as a consequence of likely translocations between non-homologous chromosomes in the interphase nucleus.

Especially regarding the species *H. regani,* we can highlight the location of nucleolus organizer regions (NORs) as a variable inter-population character. The results reveal distinct patterns of chromosome types and location that enable the *H. regani* population in the Piumhi River of the São Francisco Basin to be distinguished from the populations

in the Mogi-Guaçu and Tietê Rivers analyzed by Artoni and Bertollo (1996, 2001) and Alves et al. (2006), respectively. With these data on the gene activity of the NORs reinforced by the *in situ* chromosome location of the 18s rDNA sites (FISH), we may suggest that the colonizing *H. regani* individuals that invaded the São Francisco River Basin facilitated by the transposition of the Piumhi River (Moreira-Filho and Buckup 2005) may have originated from a population of this species that inhabits the Grande River Basin. In its turn, the last population is karyotypically distinct from other populations of this species that occur in the Upper Paraná River Basin (Fig. 4).

The results obtained with the chromosomal location of 5S gene do not currently allow any evolutionary inferences. However this cytotaxonomical marker may be important in future studies, especially regarding the number and location of this class of ribosomal DNA in *Hypostomus*.

Besides the identification of a population of *H. regani* belonging to the native ichthyofauna of the Piumhi River basin, originally founded from the ichthyofauna of the Upper Paraná River, we also verified the introduction of an exotic species in the São Francisco River Basin, with unpredictable consequences for the homeostasis of this environment.

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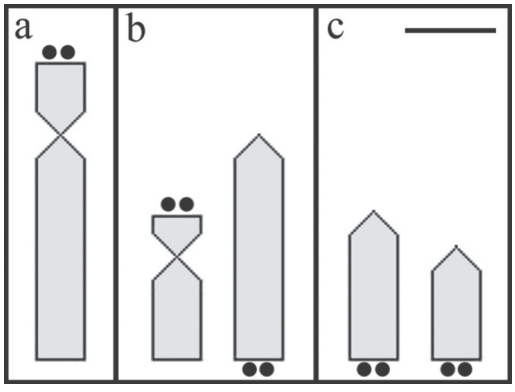

Figure 4. Idiogram of chromosomes bearing nucleolus organizer regions labeled by silver nitrate, comparing *Hypostomus regani* populations from the Piumhi River in the Upper São Francisco Basin **a** and Mogi Guaçu **b** and Tietê **c** Rivers in the Upper Paraná Basin. Bar = 1 μm.

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