

Changes in the numbers of chromosomes and sex determination system in bushcrickets of the genus *Odontura* (Orthoptera: Tettigoniidae: Phaneropterinae)

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Abstract. Chromosomes of the males of five species of *Odontura*, belonging to the subgenera *Odontura* and *Odonturella*, were analyzed. Intensive evolution of the karyotype was recorded, both in terms of changes in the numbers of chromosomes (from 2n = 31 to 27) and the sex chromosome system (from X0 to neo-XY and X0 to neo-X₁X₂Y). Karyotype evolution was accompanied by tandem autosome fusions and interspecific autosomal and sex chromosome differentiation involving changes in the locations of nucleolar organizer regions, NORs, which were revealed by silver impregnation and confirmed by FISH using an 18S rDNA probe. *O. (Odonturella) aspericauda* is a polytypic species with X0 and neo-X₁X₂Y sex determination. The latter system is not common in tettigoniids. It possibly originated by a translocation of a distal segment of the original X chromosome onto a medium sized autosome, resulting in a shortened neo-X₁ and a metacentric neo-Y. The remaining autosome homologue became the neo-X₂ chromosome. This shift from X0 to neo-X₁X₂Y is supported by the length of the X chromosome and location of the NOR/rDNA.

INTRODUCTION

Bushcrickets (Tettigoniidae Krauss, 1902), constitute a large orthopteran family, which is divided into more than 20 subfamilies (Gorochoy, 1995). Among these, the katydid (Phaneropterinae Burmeister, 1838) subfamily has more than 2300 species and at least 339 genera, distributed worldwide (Eades et al., 2010). At least 14 tribes are distinguished within this subfamily. The first systematic study of Phaneropterinae is that of Brunner von Wattenwyl (1878), who is also the author of the first revision (Brunner von Wattenwyl, 1891). Since this revision there have been no other comprehensive classifications of this subfamily. Brunner von Wattenwyl (1878) includes the genera of Phaneropterinae with very short forewings in the tribe Odonturini (= Odonturae). Jacobson (1905) distinguished a new tribe within Phaneropterinae, the Barbitistini, also for genera with very short forewings, although he did not list the genus *Odontura* Rambur, [1838]. Many scientists did not acknowledge this difference and considered the Barbitistini to be a junior synonym of Odonturini. The reduction of wings, however, has occurred quite often in the diversification of insects (e.g. Roff, 1990) and may have occurred more than once in the Phaneropterinae. Therefore, other orthopterologists treat Barbitistini and Odonturini as separate groups (Otte, 1997; Eades et al., 2010). Although, the Barbitistini seem to be monophyletic (Ullrich et al., 2010)

this may not be the case for Odonturini, which could be an assemblage of genera of short-winged forms that are only distantly related (Eades et al., 2010).

Among the eight genera distinguished in the tribe Odonturini, the genus *Odontura* is the only Palaearctic member, whereas the others occur throughout the world. *Odontura* is a western Mediterranean and North African genus including two subgenera, *Odontura* with 17 species and *Odonturella* Bolivar, 1900 with one species (Eades et al., 2010).

Comparative cytotaxonomic studies of 160 species/subspecies of 52 genera and 13 tribes of Phaneropterinae from the Palaearctic, South America, India, Africa, and Australia have yielded a range in male chromosome numbers (2n) of between 16 with neo-XY and 33 with X0 sex determination mechanisms. In some of these species (more than 70 species/subspecies in 31 genera) the karyotype consists of 31 (male) and 32 (female) acrocentric chromosomes, with an X0 (male) and XX (female) mechanisms, which seems to represent the plesiomorphic condition for Phaneropterinae and all Tettigoniidae (White, 1973; Ferreira, 1969; Warchałowska-Śliwa, 1998). The Barbitistini are cytologically the best known group within the Phaneropterinae. Over 60 species and subspecies from nine genera of this tribe have been studied cytotaxonomically (reviewed by Warchałowska-Śliwa, 1998; see also Warchałowska-Śliwa et al., 2000, 2008). The majority of these species have 2n = 31 acro-

TABLE 1. Species of *Odontura*: where and when collected and by whom, and the number of specimens examined.

Species	Collection localities, date (specimen numbers), collector(s) and where the voucher specimens are located
<i>Odontura (Odonturella) aspericauda</i> Rambur, [1838]	Spain; population A, Prov. Malaga, Serrania de Ronda, 15 km south of Ronda, 25.vi.2004 (4 ♂, 1 ♀), leg. A. Maryńska-Nadachowska (WAR3/04, WAR4/04, WAR6/04, WAR13/04, WAR5/04) Spain; population B, Prov. Alicante, Puerto de la Carrasqueta, 30 km north of Alicante, 6.vi.2005 (3 ♂), leg. A. Maryńska-Nadachowska (WAR05.03b-1, WAR05.03b-2, WAR05.03b-3)
<i>Odontura (Odontura) macphersoni</i> Morales Agacino, 1943	Spain; Prov. Caceres, Puerto de Tornavacas, 45 km north-east of Plasencia, v.2005 (5 ♂), leg. A. Maryńska-Nadachowska (WAR05.32-01, WAR05.32-02, WAR05.32-03, WAR05.32-04, WAR05.32-05)
<i>Odontura (Odontura) stenoxypa</i> (Fieber, 1853)	Italy; Sicily, Eraclea Minoa, Riserva Nazionale Foce de Fiume del Platani, 10.iv.2010 (1 ♂), leg. A. & G. Lehmann (CH7279)
<i>Odontura (Odontura) arcuata</i> Messina, 1981	Italy; population A, Sicily, Segesta, 5.iv.2010 (2 ♂), leg. A. Lehmann (CH7277-8) Italy; population B, Sicily, Selinunte, Reserve la Pineta, 8.iv.2010 (2 ♂, 2 ♀), leg. A. & G. Lehmann (CH7280-1, and CH7283-4)
<i>Odontura (Odontura) glabricauda</i> (Charpentier, 1825)	Spain; population A, Prov. Malaga, Serrania de Ronda, 25.vi.2004 (5 ♂), leg. A. Maryńska-Nadachowska (WAR9/04, WAR10/04, WAR11/04, WAR12/04, WAR13/04) Portugal; population B, Prov. Faro, Monte da Rafoia, 26.iv.2007, 3.v.2007 (1 ♂, 1 ♀), leg. A. & G. Lehmann (CHX325, CH6907) Portugal; population C, Prov. Faro, Rocha da Pena, 29.iv.2007 (1 ♂), leg. A. & G. Lehmann (CH6906)

centric chromosomes in males with an X0/XX system of sex determination and this karyotype is considered as basic/ancestral for tettigonids (e.g. White, 1973; Warchałowska-Śliwa, 1998). Most of these species tend to be karyologically conservative at the generic level.

The karyotypes of only four species of *Odontura* have been studied. In these species the diploid number (2n) of chromosomes (and fundamental number of arms, FN) is reduced to 2n = 26 with X0 and neo-XY sex chromosome mechanisms (Matthey, 1948; Alicata et al., 1974; Messina, 1981). Previously a neo-XY sex-chromosome system derived from the ancestral X0/XX was recorded in ten phaneropterid species (including *Odontura*; Dave, 1965; White et al., 1967; Ferreira, 1969, 1976; Alicata et al., 1974; Messina, 1981; Warchałowska-Śliwa & Bugrov, 1998; Webber et al., 2003), whereas a neo-X₁X₂Y system was noted only in *Letana atomifera* (Dave, 1965).

This paper characterizes the karyotypes of five species of *Odontura* paying particular attention to the presence of the multiple sex-chromosome system, X₁X₂Y. Karyotypes were analysed using conventional standard (C-banding, Ag-NOR staining) and molecular (FISH) cytogenetic staining methods. This cytogenetic study was undertaken in order to obtain a better understanding of the relationships between both subgenera of *Odontura* and between *Odontura* and other short and long-winged phaneropterids.

MATERIAL AND METHODS

A total of 28 specimens of five species of *Odontura* were collected in Spain, Portugal, and Sicily (Italy) from natural popula-

tions. The localities where the specimens were collected are listed in Table 1 (the species were determined by K.-G. Heller using the key of Llorente & Pinedo, 1990). Voucher specimens are deposited in the Collections of Heller (CH), Lehmann (CL) and Warchałowska (WAR).

Chromosomal preparations were obtained from the gonads of young adults. Testes and ovaries were excised, incubated in a hypotonic solution (0.9% sodium citrate) and then fixed in ethanol:acetic acid (3 : 1). The fixed material was squashed in 45% acetic acid. Cover slips were removed by the dry ice procedure and the preparations air-dried. In the first step, slides were stained using the Giemsa-Schiff technique. C-banding was carried out using a slightly modified version of Sumner's (1972) technique. Karyotypes were reconstructed by arranging homologous chromosomes in order of decreasing size. Relative chromosome lengths of the diploid complement including the sex chromosome(s), based on five mitotic metaphase plates from males, were calculated as a percentage of total chromosome length (% TCL) according to Král et al. (2006). Chromosomes were classified on the basis of the criteria proposed by Levan et al. (1964). The silver staining method (Ag-NO₃) for localization of the nucleolus organizer regions (NORs) was performed as previously reported (Warchałowska-Śliwa & Maryńska-Nadachowska, 1992). The fluorescence in situ hybridization (FISH) technique with ribosomal 18S DNA (rDNA) and telomeric (TTAGG)_n DNA probes was performed according to Warchałowska-Śliwa et al. (2009). Spermatogonial metaphases and meiotic stages were analyzed and photographed with a Nikon Eclipse 400 microscope fitted with a CCD DS-U1 camera and equipped with sets of standard filters. The software Lucia Image 5.0 was used and images mounted in Adobe Photoshop. For each individuals, at least five spermatogonial metaphases and 15 meiotic divisions (diplotene to metaphase I) were examined. The fixed material is deposited in the Institute of Systematics and Evolution of Animals, PAS (Kraków, Poland).

TABLE 2. Number of chromosomes, sex determination system, distribution of heterochromatin bands, and location of NORs and rDNA on the chromosomes of species of *Odontura*.

Species	2n (in the male) + sex determination, FN and chromosome morphology	C-bands	Position of NOR	rDNA – FISH signal
<i>O. aspericauda</i> population A	30 + X0, FN=31 autosomes acrocentric X acrocentric	Paracentromeric thin – all chromosomes X interstitial	Two interstitial on X	X – one interstitial
population B	28 + neo-X ₁ X ₂ Y, FN =32 autosomes acrocentric X ₁ , X ₂ acrocentric, Y metacentric	Paracentromeric thin – all chromosomes X ₁ interstitial	Interstitial on X ₁ , paracentromeric on Y	X ₁ , Y
<i>O. macphersoni</i>	28 + X0, FN = 30 autosomes acrocentric X subacrocentric	Paracentromeric thin L ₁ -M/S ₇ , S ₉ – S ₁₄ , X; M/S ₇ thick L ₁ interstitial, X telomeric	Paracentromeric on M/S ₇	M/S ₇
<i>O. stenoxypa</i>	26 + neo-XY, FN = 28 autosomes acrocentric X subacrocentric, Y acrocentric	Paracentromeric thick – all autosomes and X, thin Y X telomeric in both arms	Near end of short arm on X, paracentromeric on M/S**	X – end short arm
<i>O. arcuata</i> population A, B	26 + neo-XY, FN = 29 autosomes acrocentric X, Y acrocentric	Paracentromeric thick – all autosomes and X, thin Y X telomeric	Near paracentromeric on X	X - paracentromeric
<i>O. glabricauda</i> ; population A, B and C	26 + X0, FN = 28 autosomes acrocentric X subacrocentric	Paracentromeric thin in most autosomes, seven autosomes and X thick, L ₁ , L ₂ interstitial*, one M*	Paracentromeric on M/S ₇	M/S ₇

FN – fundamental number of chromosome arms; * intra-specific variation in heterochromatin; ** secondary NOR; (L) large-sized, (M/S) medium or small-sized autosomes; 1, 2, number of pairs of autosomes.

RESULTS

Comparison of the chromosomes of five species of *Odontura* revealed differences between their karyotypes. The physical characteristics of the karyotypes, including number of chromosomes (2n), morphology of chromosomes (including the X and Y chromosomes), fundamental number of chromosome arms (FN), sex determination mechanisms, C-banding patterns, and locations of active NORs and rDNA clusters are summarized in Table 2.

Karyotypes

The males of *Odontura* species have from 27 to 31 chromosomes (2n). All autosomes are acrocentric, whereas the X chromosome is acrocentric or subacrocentric. The species/specimens analyzed have one of three types of sex chromosome determination: X0, neo-XY or neo-X₁X₂Y.

The chromosome complement of *O. aspericauda* (population A) is characterized by 2n = 31 (30 + X), FN = 31; the autosomes can be arranged into three size groups: three long (L) (from 10.3% to 9% of TCL), four medium (M) (from 7.2% to 4.5% of TCL), and eight small (S) (from 3.8% to 2.3% of TCL) pairs; the X chromosome is the largest element in the set (23.5% TCL) (Fig. 1a, b). In males of population B, at spermatogonial metaphase, 2n = 31 chromosomes, FN = 32 and there are fourteen autosome pairs and X₁X₂Y sex chromosomes. The autosomes can be divided into three size groups: two long (9.9% and 9% TCL), four medium (from 7.3% to 5.4% of TCL), and eight small (from 4.3 to 2.8% of TCL) pairs. The neo-X₁ is acrocentric and the largest element in the karyotype (17.5% TCL), whereas the acrocentric neo-X₂ is similar

in length to the medium-sized autosomes (5.4% TCL). The neo-Y is metacentric, with a more or less similar arm length, is about 1.5 times longer than neo-X₂ (8.1% TCL) (Fig. 1c–f). On the neo-X₁ there is a secondary constriction located interstitially (Fig. 1d), which is similar to the constriction in the ancestral X (population A – Fig. 1b).

In *O. macphersoni* (one population) the chromosomal number is reduced to 2n = 29 (28 + X), FN = 30. Fourteen pairs of autosomes can be arranged into two groups, two large (L) (12.5%, 10.2% of TCL) and twelve medium or small (M/S) pairs (from 7.1% to 3% of TCL), which gradually decrease in size. The X chromosome is subacrocentric and is the largest element of the karyotype, about twice as long as the first pair of autosomes (21% of TCL) (Fig. 2 a, b).

Males of *O. stenoxypa* (a single male was studied) and *O. arcuata* (two populations studied) have the same chromosome number 2n = 28 (26 + neo-XY), but differ in number of arms on the neo-X (subacrocentric and acrocentric); the FN = 29 and 28, respectively. Autosomes of both species can be divided into two size groups: two large (10.3%, 8.9% and 11.2%, 9.8% of TCL, respectively) and eleven medium or small pairs (from 7.6% to 4% and 8.0% to 4.1% of TCL, respectively), which gradually decrease in size; the neo-X is the largest chromosome (16.7% and 18.1% of TCL, respectively), whereas the acrocentric neo-Y (1.9% and 1.1% of TCL, respectively) is the smallest element in the set (Fig. 2c–f).

The lowest chromosome number, 2n = 27 (26 + X), FN = 28, was found in *O. glabricauda* (three populations studied). There are two large (11.6%, 10% of TCL) and eleven medium or small pairs of autosomes (from 7.6% to 3.4% of TCL), which gradually decrease in size. The

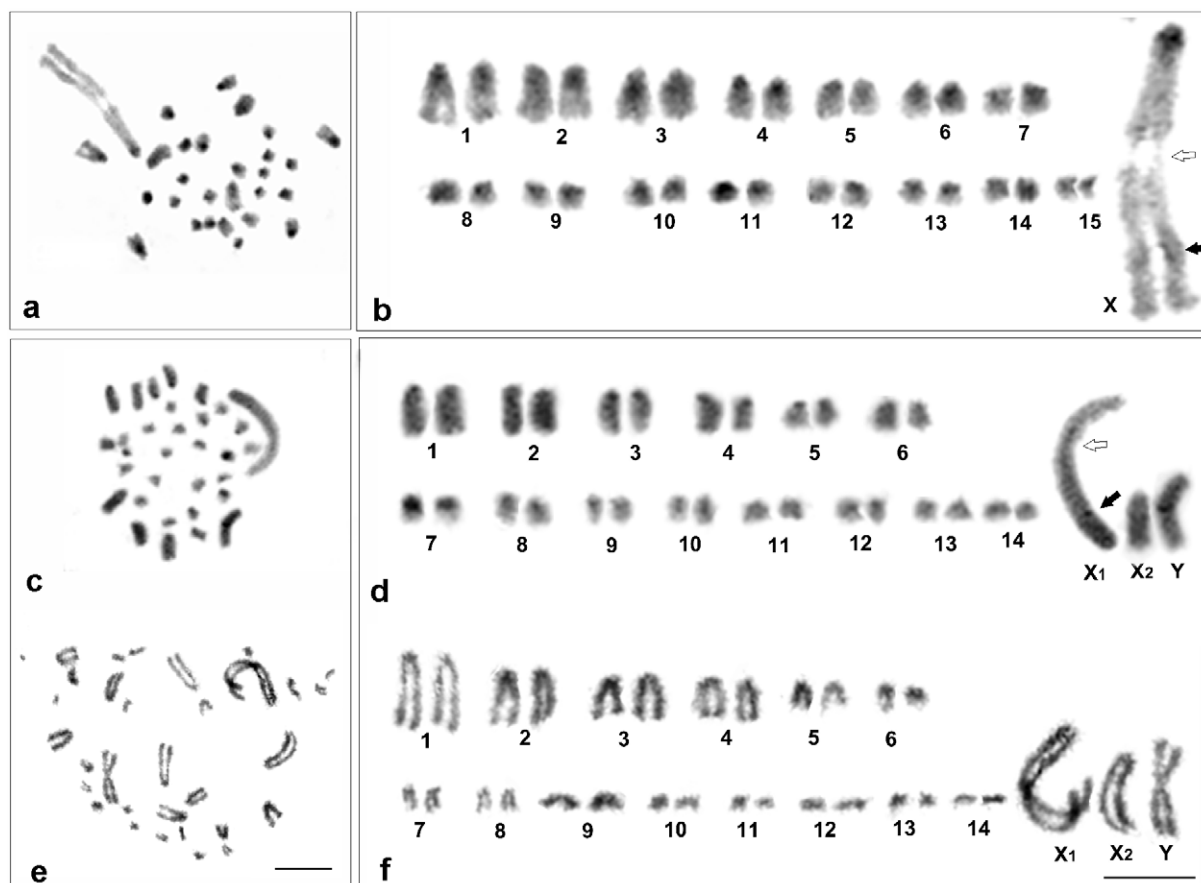


Fig. 1. Mitotic metaphases and karyotypes of male chromosome complements of *Odontura* (*Odonturella*) *aspericauda* – population A (C-banded, a, b) and population B (C-banded, c, d and Giemsa-Shiff method, e, f); open arrows indicate: (b) secondary constriction interstitially located on the X chromosome (population A) and (d) on the X₁ (population B); solid arrows indicate: (b) interstitial C-band near the distal end of the X (population A) and (d) near the distal end of the X₁ (population B). Bar = 10 µm.

subacrocentric X chromosome is clearly the largest element (26.1% of TCL) and about three times longer than the first pair of autosomes. B chromosomes (from one to four), which are supernumerary to the standard chromosome complement, were found in one out of three individuals from Portugal (population B). The length of a B chromosome is similar to that of a medium sized autosome (Fig. 2g–h). It is probably acrocentric and mitotically and meiotically unstable as observed at metaphase I and anaphase I (not shown).

C-heterochromatin

C-banding revealed some differences in number and distribution of constitutive heterochromatin blocks (C-bands) between the five species analysed (Table 2 and Figs 1 and 2). All species have paracentromeric C-bands, which vary in size in different chromosomes and species. *O. aspericauda* has thin C-bands on both autosomes and the X chromosome (Fig. 1b, d). In other species, C-bands are thick, occurring in *O. macphersoni* and *O. glabricauda* on one or seven pair/s, respectively, in *O. stenoxypa* and *O. arcuata* on all autosomes and the neo-X (Fig. 2b, d, f, g, h). Additionally, in *O. macphersoni* and *O. glabricauda* interstitial C-bands occur on one (L₁) and two of the largest (L₁, L₂) pair/s, respectively. In some individuals (one male from population A and one from C)

of *O. glabricauda* interstitial C-bands on both long and one medium pair differ in size on the two homologous chromosomes (Fig. 2b, h). In *O. aspericauda* (both populations) and *O. glabricauda*, a very weak (difficult to detect) interstitial C-band is located near the distal end of the X (Fig. 1b, d). A very thin distal band was observed on both arms of the X of *O. stenoxypa*, and on the acrocentric X of *O. macphersoni* and *O. arcuata* (Fig. 2b, d, f).

Ag-NOR staining and FISH

Silver staining revealed the presence of one active NOR in the paracentromeric region of probably the M/S₇ bivalent of *O. macphersoni* and of individuals of three populations of *O. glabricauda* (Fig. 3a, b). However, there was a second active NOR in the interstitial region of one of two large autosomes in all cells of one of five individuals from the Spanish population of *O. glabricauda* analyzed (Fig. 3b). This second NOR (not always visible) was also present on one of the small bivalents of *O. stenoxypa*. A large cluster of 18S rDNA on the M/S_{7/8} bivalent in both *O. macphersoni* and *O. glabricauda* coincides with Ag-NORs (Fig. 3c–e). In the latter species the individual with two NORs was not examined using FISH. Sex chromosome/s bear one or two Ag-NORs in *O.*

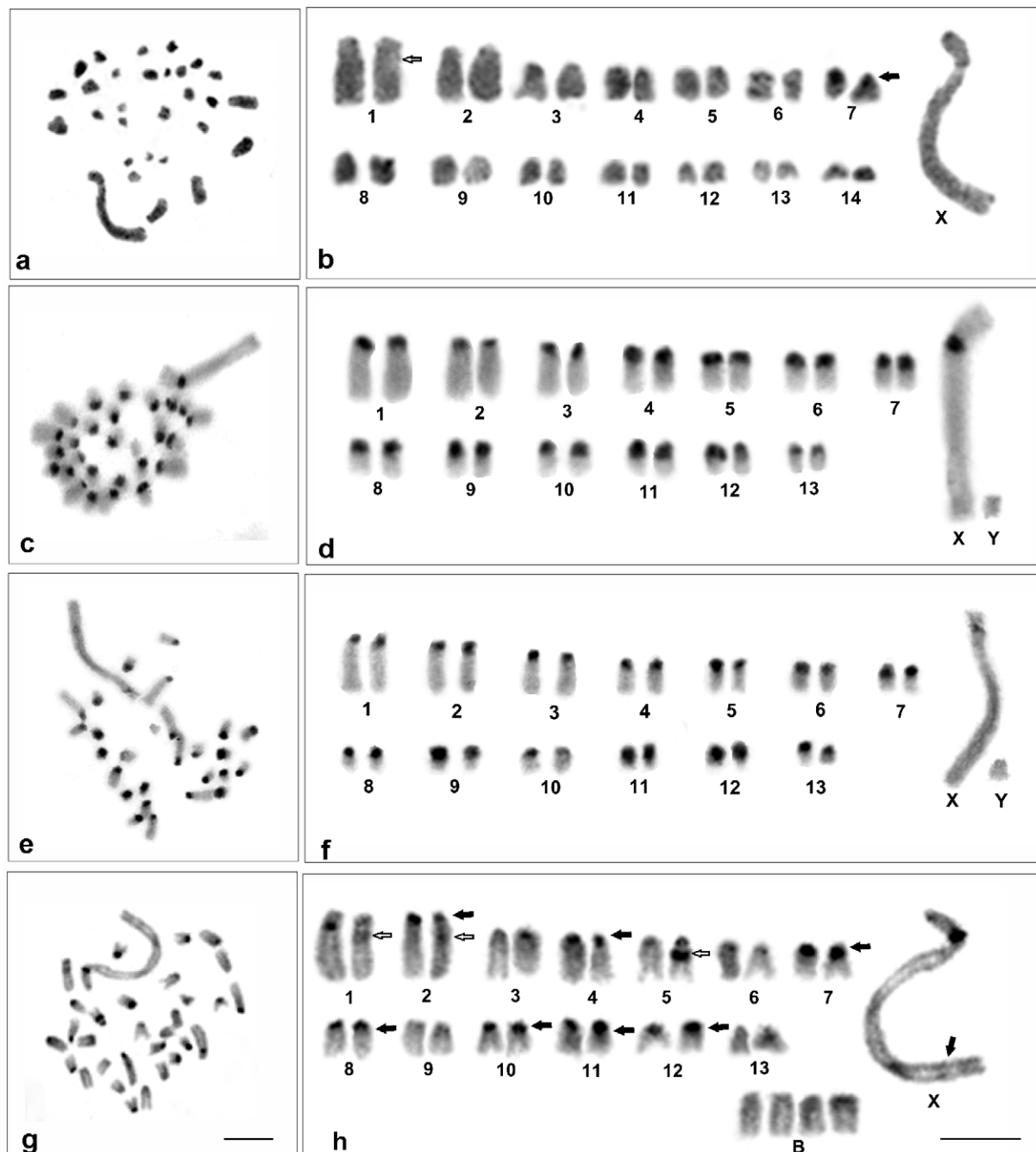


Fig. 2. C-banded mitotic metaphases and karyotypes of male chromosome complements of *O. (Odontura) macphersoni* (a, b), *O. (O.) stenoxypa* (c, d), *O. (O.) arcuata* (e, f), and *O. (O.) glabricauda* (g, h); solid arrows indicate: (b) thick paracentromeric C-bands on medium/small M/S₇ and (h) on seven pairs plus thick bands on the X; open arrows indicate: (b) interstitial C-bands on L₁ and (h) on two large and one medium pairs of homologous chromosomes that differ in size; B chromosomes (B). Bar = 10 μ m.

aspericauda, *O. stenoxypa*, and *O. arcuata* (see next section).

FISH using the (TTAGG)_n probe was performed on spermatogonial mitoses and spermatocyte nuclei at different stages of meiosis. In *O. macphersoni* (Fig. 3c), *O. glabricauda* (Fig. 3d, e), and *O. aspericauda* (population B) (Fig. 5g) FISH signals were detected at the distal ends of most chromosomes.

Analysis of sex chromosome behaviour based on classical staining and FISH

X0 system

O. aspericauda is a cytogenetically polytypic species with X0 and neo-X₁X₂Y sex determination in populations A and B, respectively. In males from population A (X0) (Fig. 4a–f), during zygotene, most of the X is heteropycnotic, whereas the distal part of the X seems to be isopycnotic (Fig. 4a). However the differences in spiralization of the X cannot be discerned at the beginning of

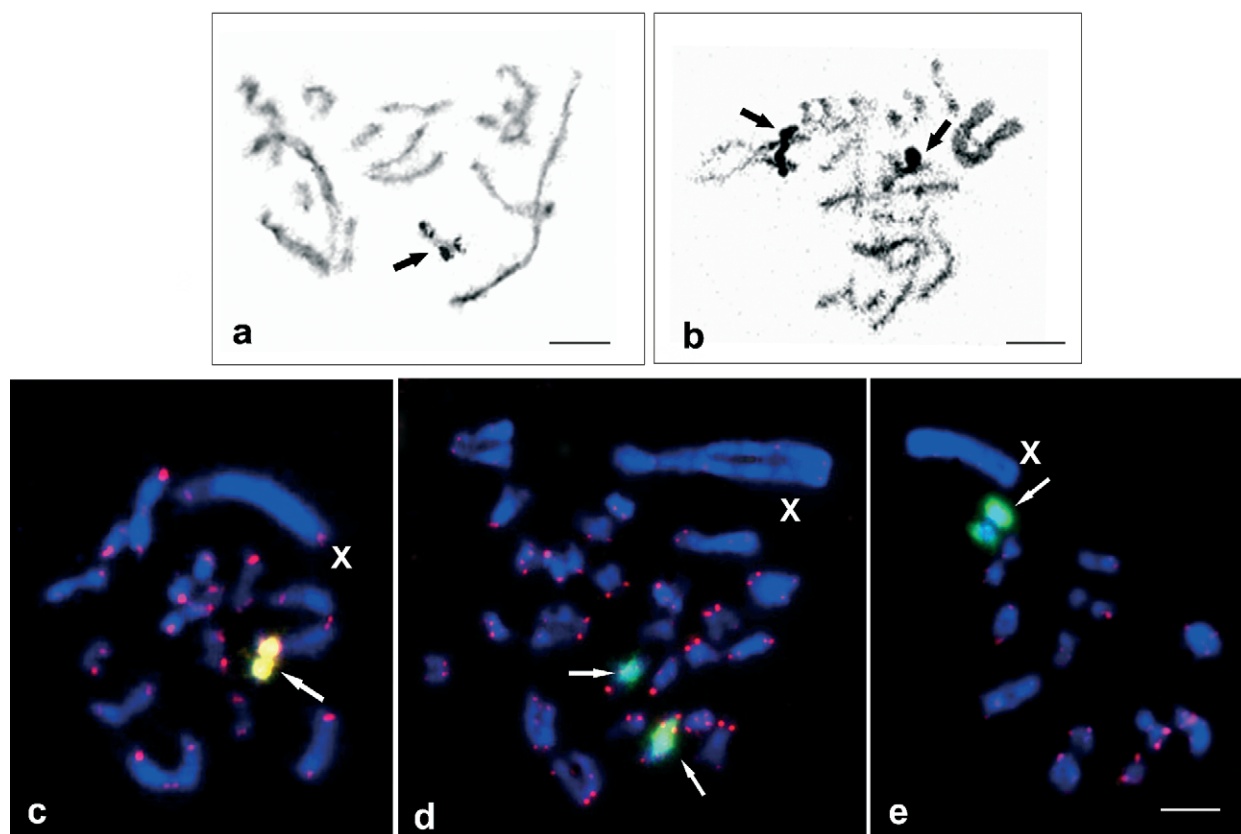


Fig. 3. Silver staining of diplotene in (a) *O. (Odontura) macphersoni*, showing the presence of one active NOR on probably M/S₇, and (b) *O. (O.) glabricauda* (only in one of five analyzed males) showing a second active NOR on the interstitial region of one large-sized autosome (arrows); (c–e) FISH using both 18S rDNA (green) and telomeric DNA (red) probes: (c) metaphase I in *O. (O.) macphersoni*, (d) spermatogonial metaphase and (e) metaphase I in *O. (O.) glabricauda* – in both species there are rDNA sites on chromosome M/S₇ (long arrows). Bar = 10 µm.

pachytene/diplotene (Fig. 4b). During spermatogonial metaphase and throughout meiosis there is a secondary constriction, interstitially located on the X (Figs 1b and 4b, c). Ag-NOR staining of spermatogonial metaphase and diplotene revealed the presence of two active NORs of different sizes in the interstitial regions of the X. The large nucleolar mass is associated with the intercalary secondary constriction, whereas the small NOR in a sub-telocentric position (not always seen) (Fig. 4d,e) probably corresponds to a heterochromatin block (Fig. 1b). However, FISH revealed only one rDNA cluster located in the interstitial region of the X, which corresponds to an active Ag-NOR (Fig. 4f).

neo-X₁X₂Y system

In individuals of *O. aspericauda* (population B) the behaviour of the neo-X₁, neo-X₂, and neo-Y sex chromosomes can be clearly observed during meiotic prophase. During early prophase (zygotene-pachytene-diplotene) only the neo-X₁ is positively heteropycnotic (Fig. 5a). At diplotene and diakinesis all sex chromosomes are connected, always in the same order, by a single terminal chiasma. During metaphase I, the neo-X₁ (probably representing most of the original X of an X₀ ancestor) is terminally associated with the “left” arm of the metacentric Y, whereas the acrocentric neo-X₂ is associated with

the “right” arm of the neo-Y (Fig. 5b). Interstitial chiasmata were not observed. For the duration of the first metaphase, the sex trivalent forms a “U”-shaped figure with neo-X₁ and neo-X₂ oriented towards one pole and the neo-Y towards the other (Fig. 5c). After anaphase I, two types of metaphase II complements are formed, with 16 chromosomes (14 + neo-X₁ neo-X₂) and 15 chromosomes (14 + neo-Y), respectively (Fig. 5d). Abnormal metaphase IIs were not found. The neo-X₁ is stained homogeneously during early meiotic prophase after C-banding. During mitosis (Fig. 1d) and pachytene/diplotene, a small C-banded region was detected in an interstitial position on the neo-X₁. Males from this population have two NORs, one located interstitially on the neo-X₁ (in the secondary constriction) and the other (smaller in size) on one arm of the neo-Y (near the centromere) (Fig. 5e). rDNA-FISH signals (coincident with active NORs) occur in the interstitial region of the neo-X₁ and (of low intensity) on one arm of the neo-Y in a sub-telocentric position (Fig. 5f, g).

neo-XY system

The neo-XY system was found in *O. stenoxypa* (Fig. 6a–f) and *O. arcuata* (Fig. 7a–g). In both species, regardless of the morphology of the neo-X (subacrocentric or acrocentric, respectively), the neo-Y is much smaller than

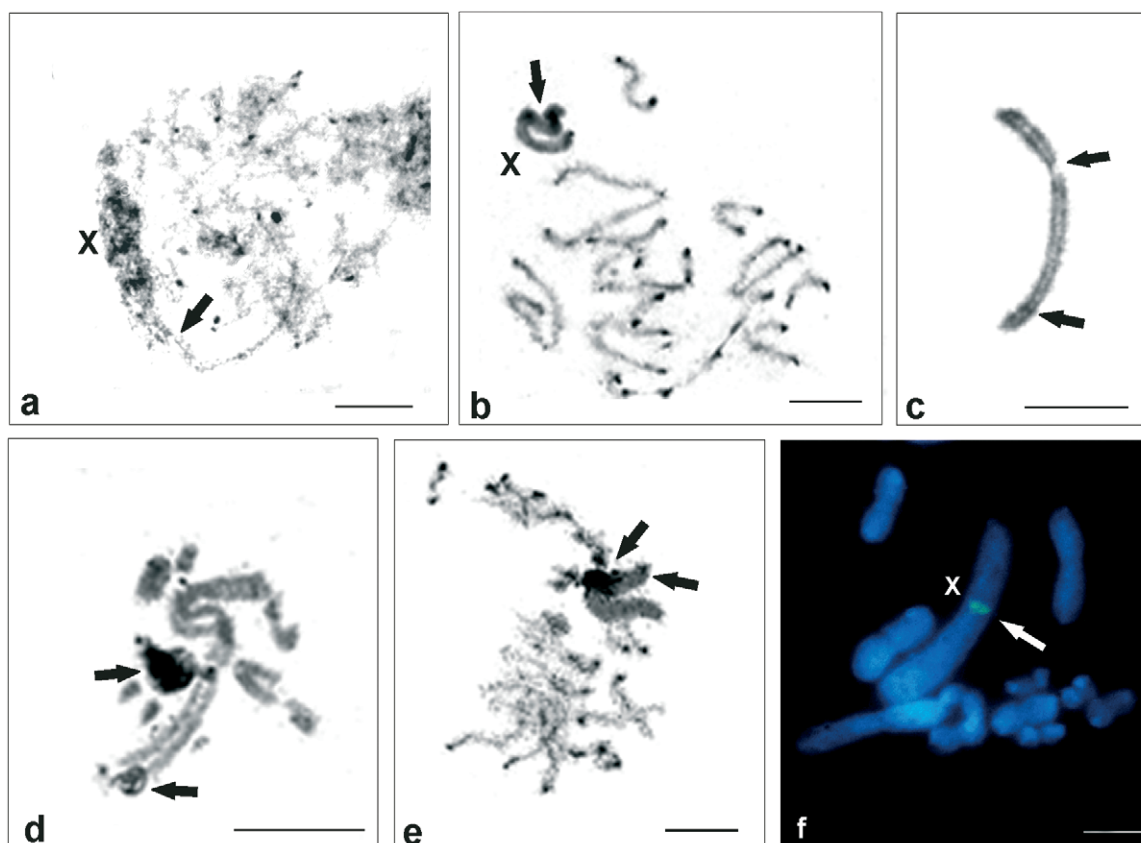


Fig. 4. The X chromosome of males of population A (X0) of *O. (Odonturella) aspericauda*; (a) zygotene, arrow indicates isopycnotic part of the X chromosome, (b) diplotene with heteropycnotic X, (c) morphotype of the X, arrows indicate secondary constriction and interstitial C-band located near the distal end; silver staining (d) incomplete spermatogonial metaphase and (e) diplotene with two active NORs (arrows); (f) rDNA cluster in interstitial region of the X identified by FISH using an 18S rDNA probe (arrow). Bar = 10 µm.

the smallest pair of autosomes (Fig. 2c–f). Silver staining showed that during pachytene, sex chromosomes form a ring or loop. In this case one of the terminal parts of both sex chromosomes becomes clearly synapsed, whereas the association between the remaining parts is asynaptic (Figs 6a and 7b). After pachytene, the sex chromosomes gradually separate and show a characteristic end-to-end association best seen at metaphase I (Figs 6b, c and 7c, e). During diakinesis/metaphase I of *O. stenoxypa* (only one individual was examined) the neo-X and neo-Y are joined by the telomeric part of the short arm of the neo-X and by the telomeric part of only one chromatid of the neo-Y (Fig. 6b, c). But in males of *O. arcuata* (there were no differences between the populations studied) the neo-X is associated with the neo-Y by its proximal or telomeric ends (Fig. 7c, d), often forming a loop-like “parachute” bivalent (Fig. 7e). Analysis of metaphase I (30 cells per individual from every population) indicates that these three types of associations occur in the same proportions. In most cells, during metaphase I, the neo-X and neo-Y begin to separate earlier than the autosomes and in a few nuclei the chromosomes are univalent. At metaphase II in both species there are 14 chromosomes, including the neo-X or the neo-Y, respectively (Figs 6d and 7f). In *O. stenoxypa*, an active NOR is located close to the end of the short arm of the neo-X. In *O. arcuata*, the NOR is

near the centromere of the acrocentric neo-X (Figs 6e and 7b). NOR activity was not detected in the neo-Y. In both species the rDNA-FISH signal is coincident with the active NOR visualized by Ag-NOR staining on the neo-X (Figs 6f and 7g).

DISCUSSION

The aim of this study was to make a contribution to the systematics of Odonturini, for which, beside morphology, little information exists. *Odontura* is karyologically an unusually diverse genus. The chromosomal complement is very variable in both diploid chromosome number and sex determination mechanism.

Cytogenetic characterization and karyotype evolution

The modal chromosome number in Odonturini is, as in most tettigoniids, $2n = 31$ in males, with acrocentric chromosomes and a X0/XX sex mechanism (e.g. White, 1973; Ferreira, 1977; Warchałowska-Śliwa, 1998). The pattern of chromosome evolution in species of the genus *Odontura* is interesting. The ancestral chromosome number and sex chromosome mechanism, $2n = 31$, X0 (FN = 31), found in Spanish *O. aspericauda* (population A), is reduced to $2n = 29$, X0 (FN = 30) in *O. macphersoni* as a result of one tandem fusion between autosomes. Three species, i.e. *O. glabricauda* (this work) and the earlier

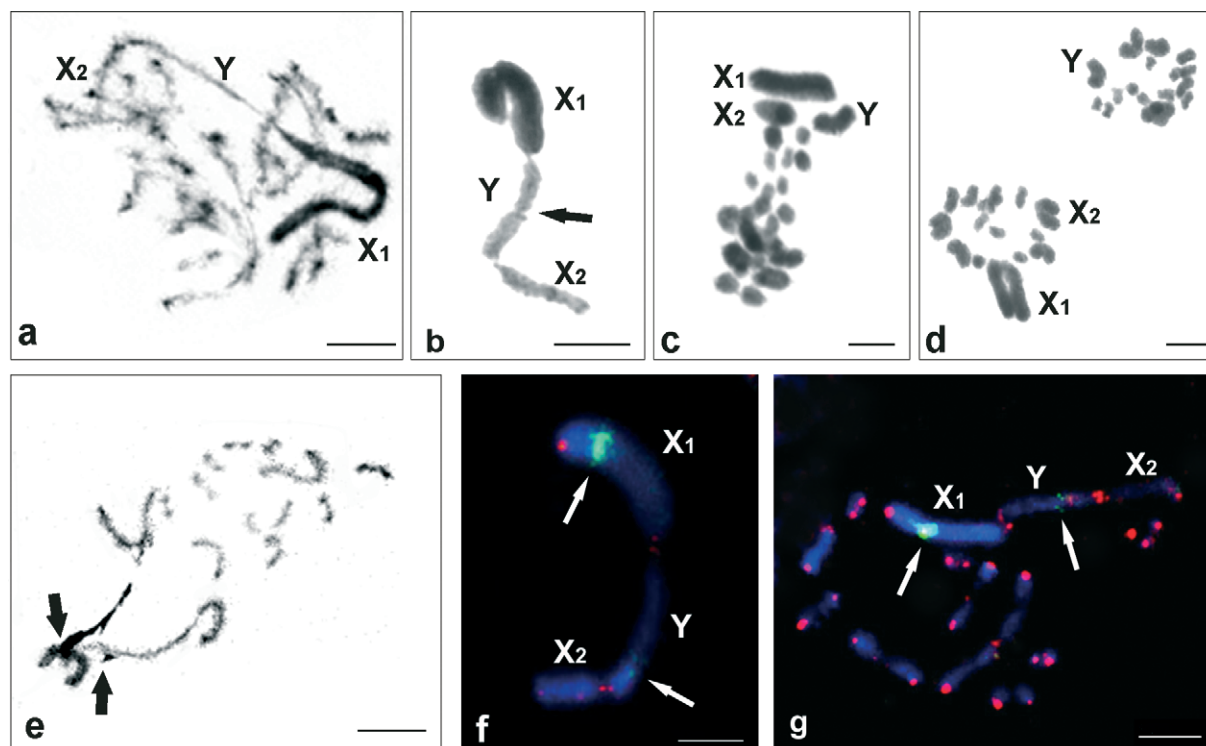


Fig. 5. The neo- X_1X_2Y system of males of population B of *O. (Odonturella) aspericauda*; (a) pachytene with positively heteropycnotic X_1 , (b) selected sex trivalent terminally associated, arrow indicates centromere on metacentric neo-Y, (c) anaphase I with trivalent, the neo- X_1 and neo- X_2 oriented to one pole and neo-Y to the other, (d) anaphase II with two types of secondary spermatocytes with 16 chromosomes (14 + neo- X_1 neo- X_2) and 15 chromosomes (14 + neo-Y); (e) silver staining of diplotene with NORs (arrows); FISH using both an 18S rDNA (green) and telomeric DNA (red) probe, (f) selected trivalent and (g) metaphase I with rDNA signals on one interstitial region of the neo- X_1 and (of low intensity) on one arm of the neo-Y in a subtelocentric position (arrows). Bar = 10 μ m.

studied *O. calaritana* and *O. maroccana* (Matthey, 1948; Alicata et al., 1974; Messina, 1981) show the next step in the reduction in the number of chromosome to $2n = 27$, X_0 (FN = 27 or 28 in different species). In all these cases, one and two tandem fusions changed the basic karyotype, however, the autosomes by subsequent inversion remain acrocentric. Analysis of the mean relative lengths of autosomes shows that the change in chromosome number in *O. macphersoni* is a result of a fusion between the first and one of the small pairs of autosomes. Two fusions occurred in *O. glabricauda*: (1) the first pair with one of the small pairs and (2) the second pair with the other small pair of autosomes. The presence of interstitial C-bands on the large autosomes of both species confirms that a tandem fusion resulted in the reduction in the number of chromosomes. These bands may represent the residual heterochromatic material from the small autosomes incorporated into the large autosomes. Terminal locations of the hybridization signals, revealed by using FISH with a telomeric probe, indicate that the telomeres are composed of $(TTAGG)_n$ repeats, as found in other Orthoptera (e.g. López-Fernández et al., 2004; Warchałowska-Śliwa et al., 2009). Pericentric inversions that modify the position of the centromere constitute another common mode of karyotype evolution within Phaneropterinae. This type of aberration has changed the morphology of the ancestral acrocentric X to subacrocentric

X in *O. macphersoni*, *O. glabricauda* (X_0) and *O. stenoxypa* (neo-X). A similar type of translocation, i.e. a biarmed X chromosome (subacro/submeta/metacentric), is reported in some species of the long-winged phaneropterids and of Barbitistini belonging to the genera *Isophya*, *Poecilimon*, and *Leptophyes* (see review in Warchałowska-Śliwa, 1998; Ferreira & Mesa, 2007; Warchałowska-Śliwa et al., 2008).

The X_0/XX sex determination found in the vast majority of Phaneropterinae is undoubtedly the initial condition for this group. It is worth mentioning that the size of the ancestral X chromosome of *Odontura* species is noticeably larger (from 26.1% to 16.7% of TCL) than the X in species of Barbitistini (from 14.9% to 13.8% of TCL) but more similar to that in other phaneropterid species of the genera *Holochlora*, *Ducetia*, *Elimaea*, *Phaneroptera*, *Tylopsis*, *Altihoratosphaga*, *Eutycorypha* and *Lunidia* (from 22.1% to 14.2% of TCL). Thus, the relatively shorter X chromosome of Barbitistini may be a specific character of this group.

In Phaneropterinae, heterochromatin analyses using C-banding and NOR Ag-staining have been used in comparative studies of species of the same genus (e.g. Warchałowska-Śliwa et al., 2008). A comparison of the C-bands of five species of *Odontura* revealed discrete differences between species, showing that the C-banding pattern and amount of heterochromatin in species of the

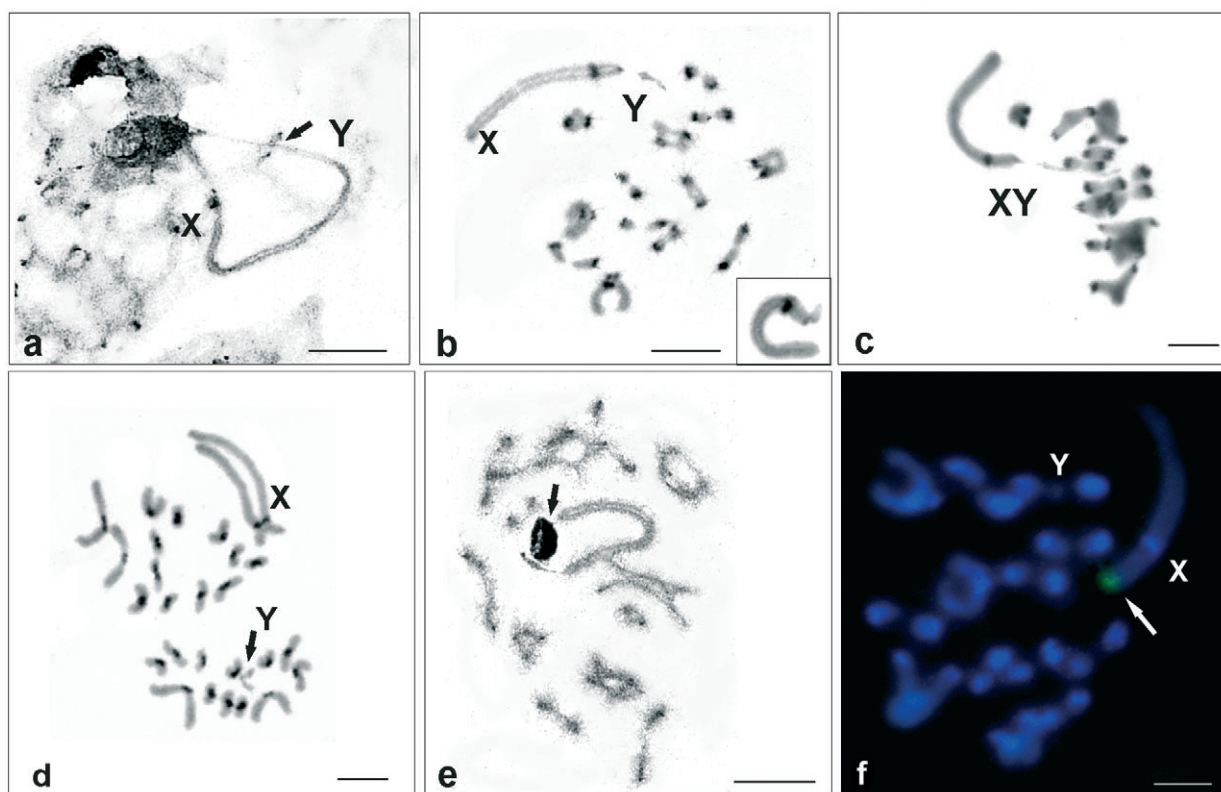


Fig. 6. The neo-XY system in males of *O. (Odontura) stenoxypa*; silver staining (a) pachytene/zygotene, sex chromosomes form a ring with a chiasma (arrow), C-banded (b) diakinesis and (c) metaphase I, subacrocentric neo-X and acrocentric neo-Y connected by telomeric segments, (d) metaphase II with 14 chromosomes (13 + neo-X and 13 + neo-Y), (e) active NOR located close to the end of the short arm of the neo-X, (f) rDNA-FISH signal on the neo-X (arrow). Bar = 10 μ m.

subgenus *Odontura* are more similar than they are to those of *O. aspericauda*, a member of the subgenus *Odonturella*. The karyotypes of five *Odontura* species have NORs located on two different chromosomes. In *O. macphersoni* and *O. glabricauda* a single NOR occurs on the medium/small autosome (M/S₇). However, in specimens from population A (X₀) of *O. aspericauda*, two NORs occur only on the X chromosome, whereas in specimens from population B (neo-X₁X₂Y), NORs are observed on both the neo-X₁ and neo-Y chromosomes. In *O. arcuata* and *O. stenoxypa*, a single NOR occurs on the neo-X. This is the first case of an unusual translocation of an NOR in sex chromosomes in tettigoniids. Data on the number and chromosomal location of NORs are fragmentary in Phaneropterinae and are only available for 23 species of the five genera of Barbitistini. The members of this group exhibit usually one, two or, very rarely more (three, four) active NORs located only on autosomes (Warchałowska-Śliwa & Maryńska-Nadachowska, 1992; Warchałowska-Śliwa et al., 1995, 1996, 2000, 2008; Warchałowska-Śliwa & Bugrov, 1998; Warchałowska-Śliwa & Heller, 1998). However, in other arthropods, not only autosomes but also sex chromosome/s often have NORs, for example, in spiders (Král et al., 2006), grasshoppers (Cabrero & Camacho, 2008), bugs (Grozeva et al., 2004), beetles (Galian et al., 2007), *Drosophila* (Roy et al., 2005) and other Diptera (Goday et al., 2006). Silver staining was used to evaluate the activity of

rDNA clusters. Ribosomal genes (rDNA) are useful for comparing the karyotypes of insect species at the genus level, e.g. in tiger beetles (Zacaro et al., 2004), grasshoppers (e.g. Loreto et al., 2008), and tettigoniids (Warchałowska-Śliwa et al., 2009; Hemp et al., 2010). In *Odontura*, the 18S rDNA loci revealed by FISH are coincident with active NORs visualized by Ag-NOR staining. The inter-specific variation in the chromosomal location of rDNA (analysed by FISH) and NOR activity (revealed by Ag-NOR staining) may be due to different mechanisms: structural chromosome rearrangements (translocations or inversions), ectopic recombination or translocation of rDNA repeats to new locations (see Cabrero & Camacho, 2008). However, as the present data cannot be used to test these hypotheses more species and individuals need to be analysed.

Structure and evolution of the neo-X₁X₂Y

The neo-XY/XX, neo-X₁X₂Y/X₁X₁X₂X₂ and neo-X₁X₂0/X₁X₁X₂X₂ types of sex determination are recorded for about 8% of orthopteran species (e.g. White, 1979; Blackman, 1995; Warchałowska-Śliwa, 1998; Mesa et al., 2002).

The comparative karyotype analysis of two Spanish populations of *O. (Odonturella) aspericauda* demonstrates polytypism and provides an insight into the chromosomal evolution of this species. Possible rearrangements involved in the origin of the neo-X₁X₂Y sex chromosome system directly from the X₀ system are

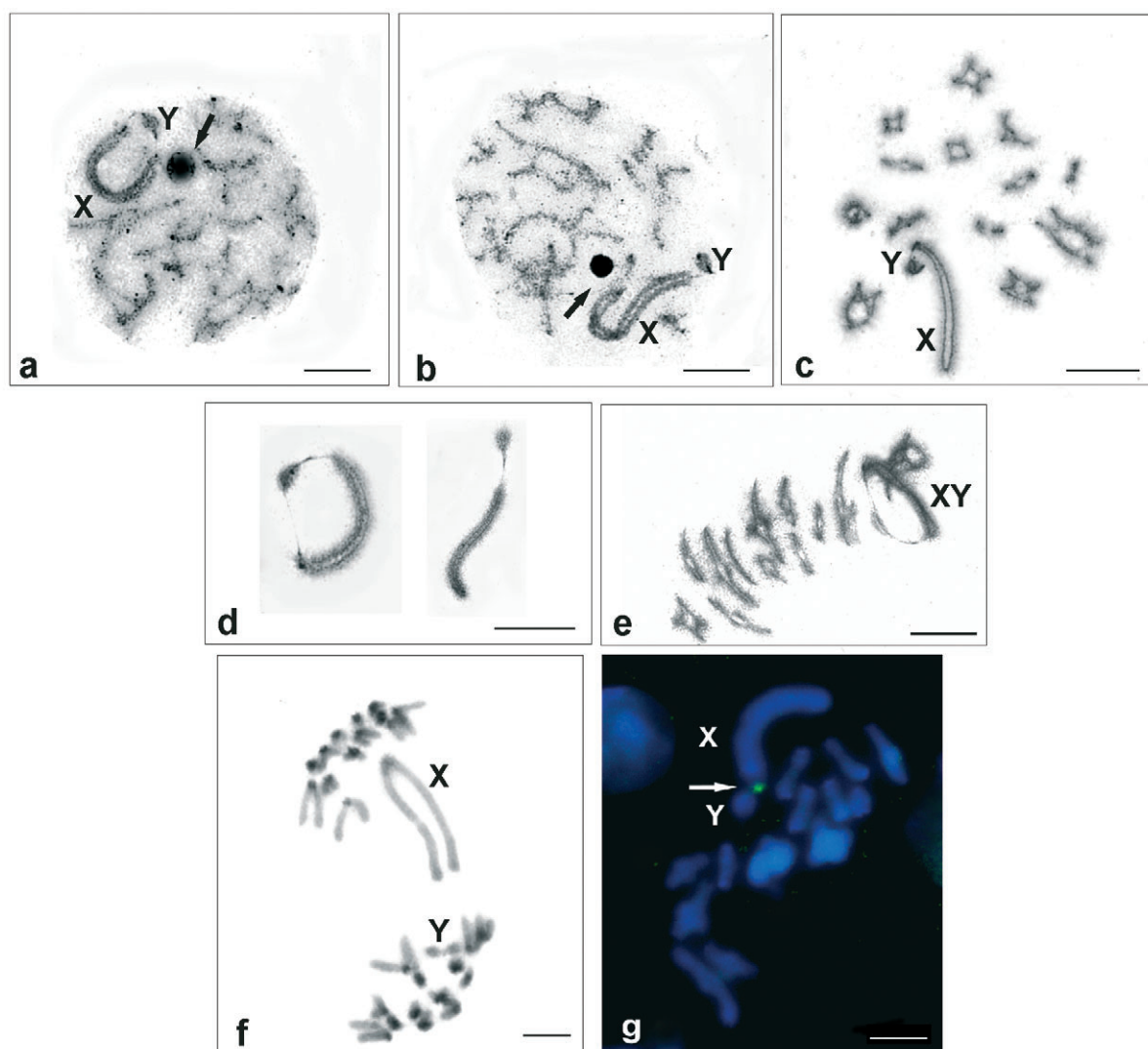


Fig. 7. The neo-XY system in males of *O. (Odontura) arcuata*; silver staining, (a) zygotene sex chromosomes form a ring or (b) end-to-end association with active NOR near centromere of acrocentric neo-X (arrows), (c) metaphase I, (d) two selected sex chromosome pairs associated by telomeric or proximal ends (repectively) and (e) metaphase/anaphase I – neo-X associated with neo-Y by both ends, forming a loop-like “parachute” bivalent; (f) two metaphase II with 14 chromosomes (13 + neo-X and 13 + neo-Y); (g) diakinesis, rDNA-FISH signal on the neo-X (arrow). Bar = 10 μ m.

shown in Fig. 8. The probable source of multiple sex chromosomes in males of population B ($2n = 28 + X_1X_2Y$) can be clearly discerned as the males differ from those of population A ($2n = 30 + X_0$) in the number of autosomes, mean length of the X chromosome and location of NOR/rDNA. The origin of neo- X_1X_2Y systems is usually explained by two successive translocations, giving a neo-XY and a subsequent translocation between the neo-Y and another autosome (e.g. Hewitt, 1979). However, in *O. aspericauda*, the shift from X_0 to neo- X_1X_2Y possibly occurred as a result of a single translocation of a distal X-chromosome segment onto a medium-sized acrocentric autosome and the remaining autosome homologue became the neo- X_2 chromosome. This hypothesis is based on the following observations: (1) the X chromosome (population A) exceeds the neo- X_1 in size (from 23.5% TCL to 17.5% TCL), (2) the neo- X_2 (population B) is similar to one of the medium-sized auto-

somes (population A), (3) two NORs occur on the ancestral X chromosome (there was one NOR on the ancestral X, the second one originated from a medium-sized NOR-bearing autosome), whereas in the neo-sex chromosomes NORs are visible on the acrocentric neo- X_1 and on one of the arms of the neo-Y (population B), and (4) almost the entire distal part of X_0 is isopycnotic (population A). Within the Barbitistini and *Odontura* (Odonturini), only one species (subgenus *Odonturella*) has two NORs on its sex chromosomes. The loss of an autosomal NOR and the acquisition of two NORs on the sex chromosome/s (populations A and B) do not exclude the possibility that NORs may have been acquired by the X chromosome several times independently. Therefore, the part of the autosome/s with NOR/s and sex chromosome translocations were initially involved in generating the X chromosome. The small NOR at the subtelocentric position on the ancestral X and neo-Y probably results from a secondary

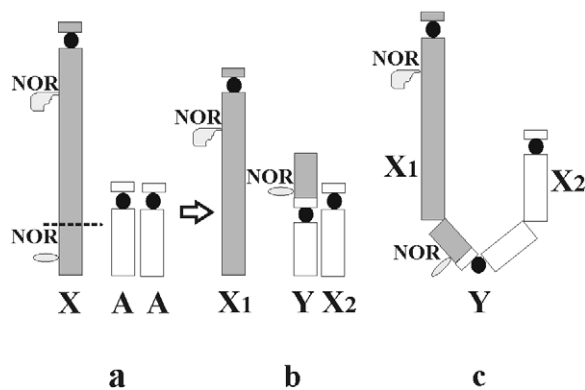


Fig. 8. A hypothetical scheme of the origin of the neo-X₁X₂Y system from X₀ in *O. (Odonturella) aspericauda* as a result of translocations between the ancestral X chromosome (X) and an autosomal pair (AA); (a) ancestral X with two NORs located in the interstitial region and a small one in a subtelocentric position, (b) neo-X₁X₂Y with NORs, one on acrocentric neo-X₁ and a second on metacentric neo-Y, (c) in metaphase I the sex trivalent consist of three elements that are always associated in the same order; (a) dashed lines indicate the break point.

acquisition. It is noteworthy that the proximal and distal parts of the ancestral X chromosome differ remarkably in morphology during prophase I. It is likely that the pattern of X chromosome condensation in *O. aspericauda* is caused by the addition of autosomal material, as in the spider *Leptoneta* sp. (Král et al., 2006). In the neo-X₁X₂Y system, the homologue of the fused autosome was transferred to the neo-Y “left” arm. Since most of the X chromosome (in the X₀ system) is heteropycnotic during zygotene-pachytene, whereas its distal part seems to be isopycnotic, it is clear why the NOR-bearing region translocated from the X to the neo-Y is not positively heteropycnotic at diplotene. It is likely that the part of the ancestral X chromosome bearing the NOR near its telomere was translocated to the centromeric part of a medium-sized autosome. As a result the neo-Y is more or less metacentric, probably approximately equal in length to the translocated part of the ancestral X but not equal to the autosome. Based on the length of the neo-Y “right” arm in comparison with neo-X₂, it is not possible to exclude that some genetic material was lost during the complicated rearrangements that resulted in the neo-sex-chromosome system. Additionally, the results indicate that in the neo-X₁X₂Y system, terminal chiasmata are always at the ends of X and Y chromosomes in the sex trivalent. Such chiasmata may facilitate congressional movements of the trivalent on the spindle (del Cerro et al., 1998). In such cases, two Xs are attached to one pole and the Y to the other pole, which results in the correct segregation of sex chromosomes at anaphase I.

Structure and evolution of the neo-XY

Over 100 cases of the neo-XY system are recorded in Orthoptera, most of which are in grasshoppers (e.g. Hewitt, 1979). In contrast, only a few instances of this system are recorded in species of Tettigoniodea, especially Phaneropterinae. In these species the neo-X is

meta- or subacrocentric produced by a centric (Robertsonian) fusion between the acrocentric X and an autosome, whereas the neo-Y is always acrocentric. In bush crickets, the single case of a neo-X in *Isophya hemiptera* (Barbitistini) was produced by tandem fusion of an autosome with the interstitial part of the original X, whereas the neo-X and neo-Y undergo a post-reductional division (Warchałowska-Śliwa & Bugrov, 1998).

In Italian populations of *O. arcuata* and *O. stenoxypa*, there exist neo-XY systems with both subacrocentric and acrocentric neo-Xs and an acrocentric neo-Y (Alicata et al., 1974; Messina, 1981). The results presented for both of the above mentioned species confirm the existence of this system, but the morphology of the neo-X differs.

In *O. arcuata* and *O. stenoxypa*, the neo-XY system probably resulted from the fusion between the ancestral X (in the X₀ system) and a small pair of autosomes bearing a NOR near the centromere. An autosome of this type occurs, e.g., in *O. macphersoni* (present study). In *O. arcuata*, the acrocentric neo-X resulted from a tandem fusion. In this case, a small part of the NOR-bearing autosome was transferred to a sex chromosome. The neo-Y, the smallest member of the set and part of a homologous autosome, resulted from the loss of the segment with the NOR and probably by additional rearrangements that resulted in the different types of associations between the neo-X and neo-Y during meiosis. However, the neo-XY system with a subacrocentric neo-X in *O. stenoxypa* could have originated independently as mentioned above. In this case, there may have been two rearrangements. First, a pericentric inversion may have changed the morphology of the ancestral X chromosome (from acrocentric to subacrocentric). Second, similar to what occurred in *O. arcuata*, a very short part of an autosome with an NOR at the end of the short arm was translocated to the X. In this species a second NOR was visible on one of the small-sized autosomes. The occurrence of this NOR was related to rearrangements in one homologue of the autosome pair, which took part in the genesis of the neo-Y.

White (1973) suggests there are two basic types of the neo-XY system, in which (1) the homologous pairing segments are restricted to distal segments of the X and Y, and (2) when they are found in blocks of heterochromatin are present in the rest of the Y. In such cases only one chiasma is formed very close to the distal end of the sex chromosome. *Chlorobalius leucoviridis* [referred to as *Yorkiella picta* (Listrosclidinae)] and *Theudoria melanocnemis* (Phaneropterinae) have one or two chiasmata between the neo-Y and neo-X that are not located distally (White et al., 1967). On the other hand, in *Neocallicrania selligera* (referred to as *Callicrania seoanei*), a species of Morabinae P45b (White et al., 1967; White, 1979) and *Isophya hemiptera* (Warchałowska-Śliwa & Bugrov, 1998), only one chiasma occurs at an interstitial or proximal position as a consequence of tandem fusion. Based on the behaviour of the sex chromosomes of *O. arcuata* and *O. stenoxypa* at meiosis the terminal regions of the neo-X and neo-Y synapse at pachytene and subsequently show a terminal association at metaphase I. The

pairing between sex chromosomes is restricted to very short terminally located heterochromatic segments of the neo-X and neo-Y. Similar meiotic behaviour of sex chromosomes is reported in species of Arvicolidae (Megías-Nogales et al., 2003). These mechanisms result in the appearance of loop-like or “parachute”-like associations of sex chromosomes, typical of Coleoptera (e.g. Dutrillaux et al., 2008), which require more detailed analysis.

In conclusion, this chromosomal analysis of five species of *Odontura* indicates that the karyotype of this genus has undergone intensive evolution, including changes in chromosome number and development of a neo-XY and neo-X₁X₂Y sex chromosome systems, which are very rare in tettigoniids. The present study focused on an analysis of the karyotype evolution in only five out of 18 *Odontura* species by mapping rRNA coding genes and telomeric sequences (for three species) and using classical cytogenetical methods. The karyotypes of the genus *Odontura* greatly differ from those of species of the Palearctic genera of Barbitistini (e.g. *Ancistrura*, *Barbitistes*, *Isophya*, *Metaplastes*, *Poecilimon*, and *Polysarcus*) in the numbers of chromosomes altered by tandem autosomal fusions, the relative length of the X chromosome, and systems of sex determination. Therefore, a reduction in the number of chromosomes has occurred within *Odontura* probably several times independently. A taxonomic revision of this genus together with cytogenetic studies and DNA-sequencing of more species is likely to result in a better understanding of the systematics of *Odontura* than of other groups of Phaneropterinae.

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