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The evolutionary origin of a novel karyotype in *Timarcha* (Coleoptera, Chrysomelidae) and general trends of chromosome evolution in the genus

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Abstract

In this work, we have analysed the karyotypes of six species of *Timarcha* for the first time and updated the cytological information for two additional taxa, for one of them confirming previous results (*Timarcha erosa vermiculata*), but not for the other (*T. scabripennis*). We describe the remarkable karyotype of *T. aurichalcea*, the lowest chromosome number in the genus ($2n = 18$), distinctive as well for the presence of an unusual chiasmatic sexual bivalent hitherto unreported for *Timarcha*. This study increases the number of species studied cytologically in this genus to forty. Additional cytogenetic analyses are performed on several species, including Ag-NOR staining and fluorescent *in situ* hybridization (FISH) studies with ribosomal DNA probes. Karyotype evolution is analysed by tracing different karyotype coding strategies on a published independent phylogenetic hypothesis for *Timarcha* based on the study of three genetic markers. The implementation of a likelihood model of character change optimized onto the phylogeny is tentatively used to detect possible drifts in chromosome changes. These analyses show that karyotype is conservative in the evolution of the genus and that there is an apparent trend to reducing chromosome number. Cytological and phylogenetic data are used to explain the evolutionary origin of the karyotype of *T. aurichalcea* by two centric fusions involving one pair of acrocentric autosomes and the sexual chromosomes.

Key words: Ag-NOR banding – centric fusion – chromosome evolution – fluorescent *in situ* hybridization – Coleoptera – speciation

Introduction

The Mediterranean genus *Timarcha* with two relict species in NW North America is possibly one of the taxonomically most difficult among leaf beetles, due not only to the high number of the so-called 'species' but predominantly to the very subtle and variable characters that separate many of them. The apterous condition of these beetles determines a low capacity for dispersal and promotes a noteworthy appearance of local races and endemic species mainly in the western Mediterranean (Bechyné 1948; Warchalowski 2003). A particularly species-rich and taxonomically problematic lineage in *Timarcha* is the *Timarcha goettingensis* species complex, which includes the widely spread European nominal taxon and a high number of closely allied forms with imprecise taxonomic and geographic boundaries, mainly distributed in the Iberian Peninsula. This lineage was defined on the basis of conspicuous cytological and morphological synapomorphies (Petitpierre 1970a), and further corroborated using molecular phylogenies (Gómez-Zurita et al. 2000a,b).

To date the cytogenetic analyses have been directed to 35 taxa in *Timarcha* (Petitpierre 1968, 1970b, 1976, 1982; Dutrillaux and Chevin 1969; Petitpierre and Jolivet 1976; Chevin 1986, 1992). These studies have shown a broad range of chromosome numbers from $2n = 20$ to $2n = 44$, although the modal value of $2n = 20$ is shared by almost 60% of the taxa examined so far, mainly represented by the closely related forms of the *T. goettingensis* complex (Petitpierre et al. 1993; Petitpierre 1997).

On the other hand, in the recent years we have carried out a molecular phylogenetic study on several taxa of *Timarcha*, by using the nucleotide sequences of two mitochondrial and one nuclear genes, to obtain a consistent and grounded hypothesis of evolutionary splitting in this genus (Gómez-Zurita et al. 2000a,b,c; Gómez-Zurita and Vogler 2003; Gómez-Zurita 2004).

Herein we provide a further study on the karyotypes of six new taxa of *Timarcha*; while two additional species, *T. erosa vermiculata* and *T. scabripennis*, are reanalysed. Besides, surveys by Ag-banding, 4,6-diamidino-2-phenylindol·2HCl (DAPI) staining, and fluorescent *in situ* hybridization (FISH) with a ribosomal DNA probe (rDNA), have been developed using several *Timarcha* taxa, depending on the availability of samples, but trying to cover all major karyotype lineages. Being a main goal of this paper to ascertain the chromosomal origin of *T. aurichalcea*, the only species in the *T. goettingensis* complex with a divergent karyotype number, we have performed most analyses on this taxon plus on the sister taxa *T. perezi* and *T. fallax*, identified as such in the phylogenetic study of the genus. Ag-NOR banding and FISH analyses using ribosomal probes are suitable techniques for the study of chromosome rearrangements, since they have been already described to often involve the nucleolar organizer in beetles (e.g. Galián et al. 2002).

The whole of the chromosome data are discussed in the light of an independent molecular phylogenetic hypothesis based on the parsimony analysis of three genetic markers (Gómez-Zurita 2004). The aim here is to evaluate the existence of rough patterns of karyotype evolution in *Timarcha* and to assess the extent of convergence and the utility of chromosomal data for taxonomic and/or evolutionary studies in this genus.

Materials and methods

Chromosome preparations

Timarcha adult male specimens (Table 1) were sacrificed with acetic ether, their gonads immediately dissected and incubated for 15 min in

Table 1. *Timarcha* samples analysed and their chromosomal data

Taxon	Source	Chromosomal data	
		Male meioformula	$2n$
T. aurichalcea Bechyné	Guadalaviar, Teruel, SP	8 + neoXY	18
	Tragacete, Cuenca, SP	8 + neoXY	18
T. cornuta Bechyné	Zicavo, Corsica, FR	9 + Xy_p	20
T. gougeleti Fairmaire	Fiobre-Bergondo, A Coruña, SP	9 + Xy_p	20
T. gr. *perezi* Fairmaire	Puerto de San Isidro, León, SP	9 + Xy_p	20
T. hispanica Herrich-Schaeffer	Cabo de São Vicente, Algarve, PO	9 + Xy_p	20
T. erosa vermiculata Fairmaire[1]	Praia de São Torpes, Sines, PO	9 + Xy_p	20
T. granadensis Bechyné	Sierra de Guillimona, Granada, SP	10 + Xy_p	22
T. scabripennis Fairmaire[1]	Yebel-Haus, Haus Range, Tanger, MO	13 + Xy_p	28

SP, Spain; FR, France; PO, Portugal; MO, Morocco.
[1]First reported in Petitpierre (1976), with identical results for *T. erosa vermiculata*, but not for *T. scabripennis* that was considered $2n = 24$.

a hypotonic solution of 1% sodium citrate, 0.005% colchicine. After incubation, the gonads were placed on a slide and squashed with insect needles on a drop of fixative solution water : ethanol : acetic acid (4 : 3 : 1). Fixation and cell spreading was done by dripping a few drops of ethanol : acetic acid (1 : 1) and glacial acetic acid onto the slides (adapted from Imai et al. 1977). Chromosome preparations were made permanent by immersion into liquid nitrogen. Standard karyotype analyses were done on 10–15 min, 2% Giemsa stained preparations. Other chromosome preparations for these and other *Timarcha* taxa were separated for the cytological study of location and activity of nuclear rDNA loci. Such data were obtained depending on sample availability and in an attempt to encompass different chromosomal classes in *Timarcha*. In the case of *T. aurichalcea* and its closely allied taxa *T. fallax* and *T. perezi*, these were subject to more intensive cytological analyses to find out the origin of the chromosomal rearrangements differentiating them.

Silver staining of nucleolar organizer regions

Active nucleolar organizer regions (NORs) in dividing cells were detected using Ag-NOR banding following the one-step protocol of Howell and Black (1980). Slides were incubated for 3–10 min on a metallic plate at 43°C with 60 μl of $AgNO_3$ 0.5 g/ml and 30 μl of a 2% gelatine and 1% formic acid solution. The preparations were rinsed in abundant distilled water, air-dried and studied under the microscope.

Fluorescent *in situ* hybridization

The FISH localization of ribosomal probes on chromosomal preparations of *Timarcha* was done based on the method by Juan et al. (1993). The probe, containing the ribosomal genes of *Drosophila melanogaster*, was biotin-labelled by nick translation using the Bionick kit (Gibco BRL, Rockville, MD, USA). Briefly, the FISH experiments consisted of a pre-treatment of the preparations with RNase [100 μg/ml in 2x SSC (300 mM NaCl, 30 mM sodium citrate, pH 7.0); 1 h at 37°C in humid chamber] and pepsin (0.005% in 10 mM HCl; 10 min at 37°C in humid chamber), followed by two 1x PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.0) rinses for 5 min at room temperature. The material was fixed for 10 min in 0.9% formaldehyde, 47 mM $MgCl_2$ in PBS, rinsed again with PBS and subsequently dehydrated in 70, 90 and 100% ethanol (5 min per treatment). About 1 μl of probe (10 ng/μl) diluted in 4 μl 60% formamide, 2x SSC and phosphate buffer (pH 7.0) was added to the preparations, covered with a 18 × 18 mm coverslip and incubated for 3 min at 80°C to denature both probe and chromatin. Hybridization took place overnight at 37°C in a humid chamber. Post-hybridization washes (after careful removal of coverslip) consisted in three rounds of 50% formamide, 2x SSC at 37°C, two rounds of 2x SSC at RT, and one final wash in 4T buffer (4x SSC, 0.05% Tween20) at room temperature. The probes were immunologically detected with subsequent steps of avidin-FITC (FITC: Fluorescein isothiocyanate; Vector Labs, Burlingame, CA, USA), biotinilated goat anti-avidin-FITC (Pierce Labs, Rockford, IL, USA) and a second round of avidin-FITC, with intermediate washes using 4T and a final wash with PBS. The samples were dehydrated in increasing concentrations of ethanol, dried, and counterstained with propidium iodide and DAPI for 5 min. Hybridized chromosomes were photographed with a Zeiss Axiophot Fluorescence Microscope (Carl Zeiss, Jena, Germany).

Phylogenetic tests

The patterns of chromosome evolution in *Timarcha* were studied by tracing different alternatives for karyotype character coding onto an independent phylogenetic hypothesis for the genus provided by a simultaneous parsimony analysis of three genetic markers (Gómez-Zurita 2004). This phylogeny is the best hypothesis available for *Timarcha* in terms of resolution, support and taxon coverage. Three alternative character coding strategies for chromosome data were compared with different degrees of information (Table 2): (i) Plain chromosome numbers (CC1), (ii) major classes of karyotypes based on chromosome relative sizes (CC2), and (iii) subdivision including positional information of the centromere for a specious lineage in the genus, the *T. goettingensis*-complex (CC3).

For each alternative we tested the phylogenetic structure of the observed character states through permutation tests. The number of character transformations required on the tree topology was compared with 999 random permutations without replacement of the original unordered character matrix to simulate the null hypothesis of absence of phylogenetic structure in the data. Significant results were interpreted as evidence for a phylogenetic conservation in karyotype characteristics.

We have tentatively explored the existence of trends in the evolution of chromosome number in *Timarcha* by applying likelihood methods to reconstruct ancestral character states. In particular we have implemented the CC1 character coding with ordered characters from highest to lowest chromosome number and used the tree topology to estimate an instantaneous rate matrix with two parameters: f, for increase in the character state (decrease in chromosome number), and b, for decrease in character state (increase in chromosome number). The parameters f and b are the average proportion of each type of change (i.e. probabilities in the likelihood model) as estimated optimizing on the tree topology the reconstruction of this character history. Multiple character states need to be coded by either increasing or decreasing chromosome number for the test to be meaningful, and this choice only conditions how the results of the test need to be interpreted. The two-parameter model used corresponds to the 'Asymmetrical Markov k-state 2 parameter model', a generalization of Lewis's (2001) 'Markov k-state 1 parameter model', the latter with occurring changes and reversals assigned the same probability. The averaged f/b rate and its significance (calculated as above) were considered as indicating potential trends in chromosome number evolution. The significance of this statistic was obtained by comparison with the distribution of the corresponding value for 999 random permutations of the original character matrix. These tests were done excluding taxa with unknown karyotypes and pruning them from the tree to allow for parameter

Table 2. Character coding strategies for the evolutionary analysis of karyotypes in *Timarcha*

Species[1]	CC1 (2*n*)	CC2	CC3[2]	Reference
T. intricata	44	A	–	Petitpierre and Jolivet 1976
T. cerdo	38?	B	–	Jolivet and Petitpierre 1992
T. calceata	30	C	–	Petitpierre 1976
T. nicaeensis	28	D	–	Chevin 1986
T. pimelioides	28	D	–	Petitpierre 1976
T. punctella	28	D	–	Petitpierre 1976; this work
T. scabripennis	28	D	–	this work
T. strangulata	28	D	–	Petitpierre 1970b
T. espanoli	26	E	–	Petitpierre 1970b
T. rugosa	26	E	–	Petitpierre 1976
T. tangeriana	26	E	–	Petitpierre 1970b
T. balearica	22	F	–	Petitpierre 1970b
T. granadensis	22	F	–	this work
T. tenebricosa	22	F	–	Petitpierre 1970b; Chevin 1985
T. metallica	20	G	–	Petitpierre 1982
T. insparsa	20	H	–	Petitpierre 1976
T. intermedia	20	H	–	Petitpierre 1970b
T. lugens	20	H	–	Petitpierre 1976
T. marginicollis	20	H	–	Petitpierre 1976
T. erosa vermiculata	20	I	–	Petitpierre 1976; this work
T. hispanica	20	I	–	this work
T. cornuta	20	J	GC1 m/sm/m/m	this work
T. cyanescens (Cap Saint Martin)	20	J	GC1 m/sm/m/m	Petitpierre 1970b
T. fallax (Alió)	20	J	GC1 m/sm/m/m	Petitpierre 1970b
T. fallax (Garraf)	20	J	GC1 m/sm/m/m	Petitpierre 1970b
T. goettingensis	20	J	GC1 m/sm/m/m	Dutrillaux and Chevin 1969; Chevin 1987
T. sinuatocollis	20	J	GC1 m/sm/m/m	Petitpierre 1970b
T. monserratensis	20	J	GC1 m/sm/m/m(var.)	Petitpierre 1970b
T. cyanescens (Anglet)	20	J	GC2 sa/sa/m/m	Chevin 1993
T. fallax (Paterna)	20	J	GC3 sm(var.)/sm/m/m	Petitpierre 1970b
T. geniculata	20	J	GC4 sa/sm/m/sm	Petitpierre 1970b
T. maritima	20	J	GC5 sa/sa/m/sm	Petitpierre 1970b; Chevin 1992
T. perezi	20	J	GC6 sa/sm/m/a	Petitpierre 1970b
T. recticollis	20	J	GC7 m/sm/m/sm	Petitpierre 1970b
T. interstitialis	20	J	GC7 m/sm/m/sm	Petitpierre 1976
T. gougeleti	20	J	GC8 m/sa/sm/sm	this work
T. aurichalcea	18	K	GC*	this work

[1]The name within brackets given for taxa cytologically polymorphic indicates the specific locality where the specimens show each karyotype.
[2]In GC karyotypes, the position of the centromere is given for first, second, third pairs of autosomes and for the X-chromosome, respectively (m, metacentric; sm, submetacentric; sa, subacrocentric; a, acrocentric; var, polymorphic).

estimation. Significant $f/b > 1$ can be interpreted as an excess of chromosome fusions; significant $0 < f/b < 1$ indicate excess of chromosome fissions; non-significant f/b or $f/b = 1$ indicate no trend whatsoever. This approach is regarded as tentative since the likelihood model is considered unrealistic for more than two character states and is also dependent on branch lengths, which are scaled to 1.0 in our application (see Maddison and Maddison 2003a). However, we use it here as an exploratory tool and the obtained results are interpreted with caution.

Character reconstruction, estimation of likelihood parameters and randomization tests were done with the software Mesquite version 1.0 and the specific module StochChar version 1.0 (Maddison and Maddison 2003a,b).

Results

Conventional staining analyses

The karyotypes of *T. aurichalcea*, *T. cornuta*, *T. gougeleti*, *T. hispanica*, *T. granadensis* and one unidentified taxon close to *T. perezi* are reported for the first time; those of *T. erosa vermiculata* and *T. scabripennis* were studied by Petitpierre (1976). *T. aurichalcea*, *T. cornuta*, *T. gougeleti* and T. gr. *perezi* belong to the *T. goettingensis* species complex, and this is corroborated by the cytological study.

The karyotypes of *T. cornuta* (Corsica), *T. gougeleti* (NW Spain) and *Timarcha* gr. *perezi* (Picos de Europa in N Spain) have three large and six small autosome pairs of which the fourth pair is acrocentric and the remaining pairs are metacentrics. They also have a rather large X-chromosome and a small y-element, as found in other taxa of the *T. goettingensis* complex (Petitpierre 1970b). These three taxa differ in the shape of first and third pair whereas the second pair is subtelocentric in all. *T. cornuta* has the first and third pair metacentric, *T. gougeleti* a first metacentric and a third submetacentric, while in *Timarcha* gr. *perezi* the first is subtelocentric and the third metacentric (Fig. 1a–c). Their meiotic formula is $9 + Xy_p$, that is, they have a 'parachute-like' achiasmatic sex–chromosome pair (Fig. 2a–b).

The karyotype of *T. aurichalcea* has $2n = 18$ chromosomes, with three large autosome pairs as in the previous taxa, but contrary to the above species lacks the acrocentric fourth pair, while the X-chromosome is the largest and the Y-chromosome is also a quite large element, clearly longer than any of the five small autosome pairs (Fig. 1d). The meiotic formula of this species is 8 + neoXY, because the sex–chromosome bivalent is an asymmetric chiasmate pair of large size and well

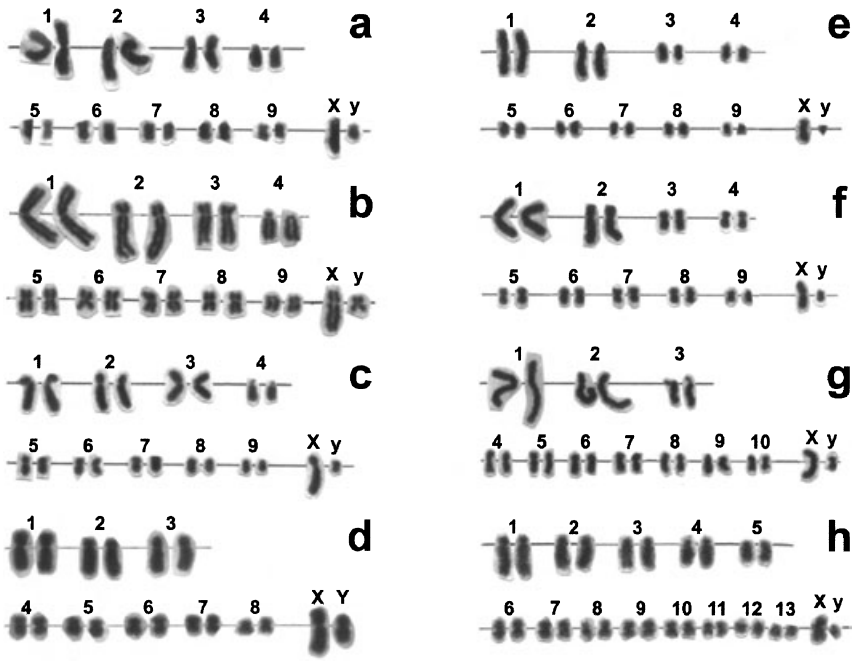

Fig. 1. Karyograms from spermatogonial metaphases or prometaphases of the species *Timarcha cornuta*, Zicavo, Corsica (a), *T. gougeleti*, Fiobre-Bergondo, A Coruña (b), *T.* gr. *perezi*, Puerto de San Isidro, León (c), *T. aurichalcea*, Tragacete, Cuenca (d), *T. hispanica*, Cabo São Vicente, Algarve (e), *T. erosa vermiculata*, Praia São Torpes, Sines (f), *T. granadensis*, Sierra de Guillimona, Granada (g), and *T. scabripennis*, Lengua de Tierra, Nador (h). Chromosomes are aligned by the position of their centromeres

differentiated from the Xy_p of the former taxa (Fig. 2c). As expected, the metaphases II showed nine chromosomes of which four are of large and five of small size (Fig. 2d).

The chromosome sets of *T. hispanica* (Cabo São Vicente) and *T. erosa vermiculata* from (São Torpes), both from southern Portugal, have $2n = 20$ and include two large autosome pairs, the first metacentric and the second subtelocentric, plus seven small metacentric autosome pairs, a medium-size metacentric X and seemingly a minute metacentric y-chromosome (Fig. 1e–f). These two karyotypes have proven to be indistinguishable by conventional Giemsa staining procedures. The meiotic formula of both taxa is $9 + Xy_p$ and again, as expected, it involves two large and seven small autosomal bivalents plus the 'parachute' sex–chromosome pair (Fig. 2e).

Timarcha granadensis, (SE Spain), shows $2n = 22$ chromosomes of which two pairs are large and similar in size and shape to those of the two previous taxa, the third autosome pair is formed of medium-size acrocentrics, the remaining seven autosome pairs are small metacentrics, the X is a fairly large metacentric and the y-chromosome the smallest metacentric element in the set (Fig. 1g). Its meiotic formula, $10 + Xy_p$, has two large and eight small autosomal bivalents besides the 'parachute' sex–chromosome pair, in agreement with the above described karyotype (Fig. 2f).

The diploid set of *T. scabripennis*, from Northern Morocco, has $2n = 28$ chromosomes, an amendment to the $2n = 24$ reported before (Petitpierre 1976). It includes five large or medium subtelocentric/acrocentric pairs and eight small metacentrics plus the smallest acrocentric pair, a medium-size X and a minute y-chromosome (Fig. 1h).

Ag-NOR staining and FISH analyses

The Ag-banding in all species of *Timarcha* studied thus far have shown only a single NOR-bearing meiotic signal regardless of chromosome number: it is found in *T. marginicollis* with $2n = 20$ (not shown), *T. granadensis* with $2n = 22$, *T. espanoli* with $2n = 26$ and *T. punctella* with $2n = 28$ (Fig. 3a–c). This result was also confirmed by additional FISH studies using the rDNA probe on different species of the genus. The rDNA probe gave a prominent FISH label in the short-arm of the acrocentric fourth chromosomes and in the counterpart metaphase I bivalent of two taxa included in the *T. goettingensis* complex, namely *T. perezi* and *T. fallax* (Fig. 4a–c). A DAPI staining yielded also a noteworthy signal in the short chromosome arms of this fourth bivalent (Fig. 4d). Similarly, other species such as *T. lugens* and in *T. espanoli* also displayed a single autosomal ribosomal locus (Fig. 4e–h).

As reported above, the karyotype and meiotic formula of *T. aurichalcea*, $2n = 18$ and $8 +$ neoXY, differs strikingly from those of the other morphologically similar taxa of the *T. goettingensis* complex, with $2n = 20$ and $9 + Xy_p$. The Ag-staining of this species chromosomes, either in diakinesis or in metaphases I, produced a remarkable signal on the asymmetric chiasmate neoXY pair (Fig. 5a–b). The rDNA probe hybridized on spermatogonial metaphase chromosomes allowed us to identify this label on the large X-chromosome only, thus excluding the Y-chromosome as a NOR-bearing (Fig. 5c).

Phylogenetic analysis of karyotype change

Figure 6 shows the three alternative character coding strategies CC1, CC2 and CC3 traced onto the phylogenetic hypothesis for the genus *Timarcha*. The deeper nodes in the phylogeny, where several chromosomally heterogeneous lineages converge, present the highest uncertainties in reconstructing their character state. Independently of the character coding used, karyotypes in *Timarcha* show a significant phylogenetic structure. The reconstruction of CC1 required 12 steps onto the phylogeny ($p = 0.001$; range in the randomization: 13–18), 15 steps for CC2 ($p = 0.001$; 19–25), and 25 steps for CC3 ($p = 0.001$; 28–39). The latter test was repeated considering only the clade affected by character recoding, i.e. the *T. goettingensis*-complex clade plus the sister taxa

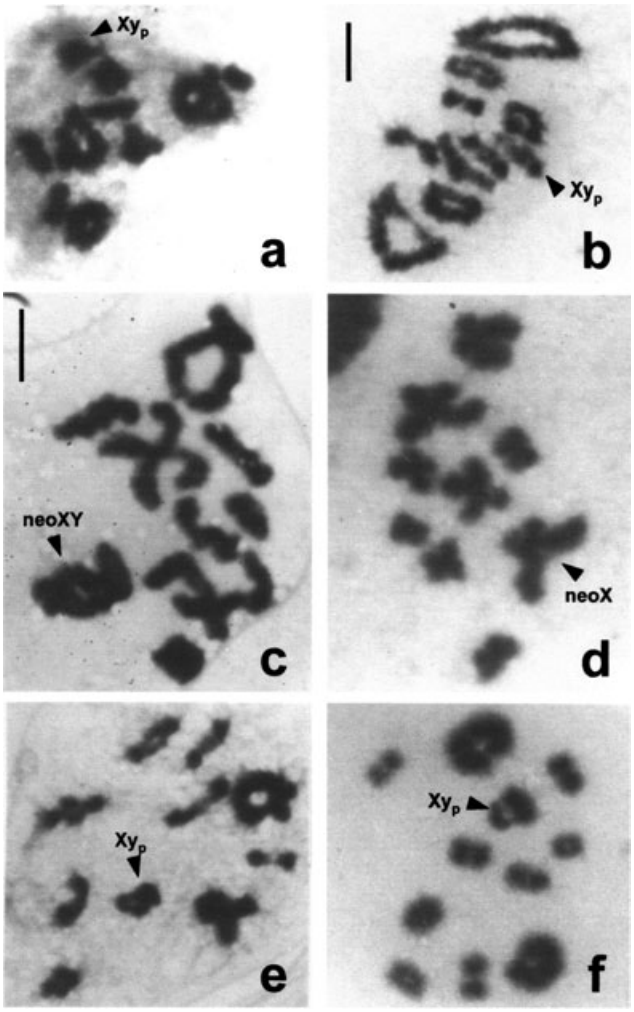

Fig. 2. Meiotic metaphases I of *Timarcha cornuta*, Zicavo, Corsica with 9 + Xy_p (a), diakinesis of *T. gougeleti*, Fiobre-Bergondo, A Coruña with 9 + Xy_p (b) and *T. aurichalcea*, Tragacete, Cuenca with 8 + neoXY (c), and metaphases I of *T. hispanica*, Cabo São Vicente, Algarve with 9 + Xy_p (e) and *T. granadensis*, Sierra de Guillimona, Granada with 10 + Xy_p (f). Arrowheads indicate the presumptive 'parachute'-like sexual bivalents Xy_p or chiasmatic neoXY bivalent, in the case of *T. aurichalcea*. Meiotic metaphase II X-class in *T. aurichalcea* (d). An arrowhead points to the clearly distinguishable sex chromosome, the largest of the complement. Scale bar = 5 μm (c, d at a higher magnification)

T. erosa vermiculata, *T. hispanica* and *T. granadensis*. For this subclade, requiring 14 character state changes, the result of the test was also highly significant ($p = 0.006$; 14–19).

The estimated parameter f/b, which provides a rough estimation of the trends in chromosome number evolution, had a value of 3.546 ($p < 0.007$). According to this figure, the number of events resulting in the reduction of chromosome number would be in the order of three to four times higher than the proportion of events increasing the number of chromosomes in *Timarcha*.

Discussion

Patterns of chromosome evolution in *Timarcha*

We provide additional evidence for the previously suggested ancestral karyotypic condition of $2n = 20$ chromosomes and

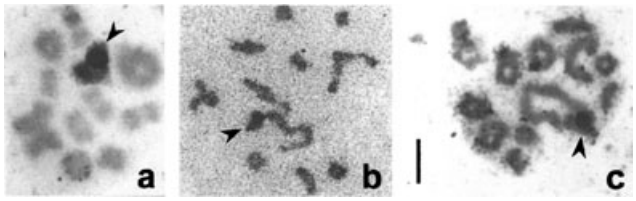

Fig. 3. Silver staining of meiotic metaphases I of *T. granadensis*, Sierra de Guillimona, Granada with 10 + Xy_p (a), *T. espanoli*, Arenales del Sol, Alicante with 12 + Xy_p (b), and diakinesis of *T. punctella*, Lengua de Tierra, Nador with 13 + Xy_p (c). An arrowhead shows the localization of the most predominant silver precipitate identifying one single active NOR per cell. Scale bar = 5 μm

9 + Xy_p male meiotic chromosome formula in *Timarcha* (Petitpierre 1970b, 1973, 1976). This hypothesis was mainly based on the agreement between the former modal karyotype for the genus and the presumed $2n = 20$ (Xy_p) ancestral chromosome constitution for the Coleoptera of suborder Polyphaga (Smith and Virkki 1978; Virkki 1994), as well as on the putative derivation of morphological characters and groups of species held by Bechyné (1948). This interpretation is in agreement with the character optimization considering the plain chromosome number (CC1) which reconstructs the inferred state of the deeper nodes in the *Timarcha* lineage, excluding the North American taxa, as $2n = 20$ (Fig. 6).

The karyotypic diversity in *Timarcha* is rather high considering the range in diploid chromosome numbers from 18 to 44 (Petitpierre et al. 1993; Petitpierre 1997). However, we have shown that there is a marked phylogenetic conservatism in chromosome characteristics in the different lineages of the genus. This is confirmed by the significantly lower number of character step changes suggested by the phylogeny of the genus than would be expected if those changes had occurred at random. Closely related species share similar karyotypes, as it is more evident in the *T. goettingensis*-complex ($2n = 20$) or in the lineage of *T. rugosa*, with all species sharing $2n = 26$. With rare exceptions, it is possible to make predictions about karyotype structure in *Timarcha* according to phylogenetic and genetic relatedness, as we predict will be the case for the pair of taxa *T. coarcticollis* and *T. riffensis*, that should display a karyotype similar to that of *T. insparsa* ($2n = 20$), or for *T. maroccana* that probably has a higher chromosome number, like its other North African relatives.

The study of forward/backward rates in chromosome number changes using the phylogeny of *Timarcha* as backbone for the estimation of a likelihood model of character change resulted in a significant excess of karyotype reduction events over increases in the number of chromosomes. The same analysis was done excluding the two basal taxa, *T. cerdo* and *T. intricata*, those with the highest chromosome numbers, 38 and 44, respectively. This lineage, being basal to the remaining *Timarcha*, could have the effect of overestimating events leading to reduction in chromosome number. The exclusion of these data resulted in an estimated f/b character change rate of 2.101 ($p < 0.005$), almost half the value obtained from the analysis with all data, but still supporting an overall trend towards a reduction in chromosome number.

The trend towards a decrease in the number of chromosomes through fusion events in the evolution of *Timarcha* is counter to

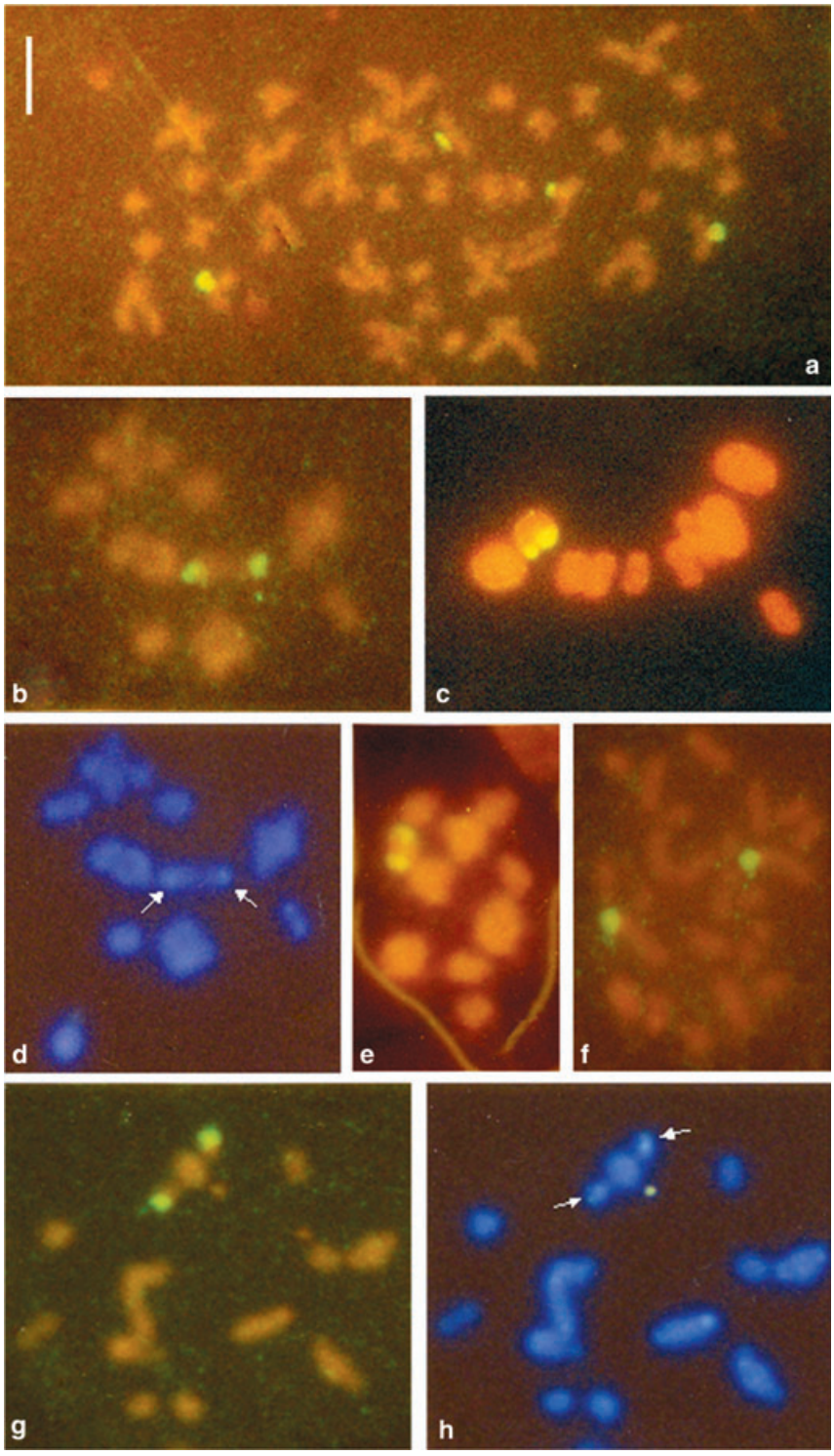

Fig. 4. Localization of rDNA gene clusters using fluorescent *in situ* hybridization in four meiotic metaphases II of *Timarcha perezi*, Puerto de Oncala, Soria where only the acrocentric fourth chromosomes are labelled (a), meiotic metaphase I chromosomes of *T. perezi*, Puerto de Oncala (b), *T. fallax*, Alió, Tarragona (c), *T. lugens*, Pico Veleta, Granada (e), and *T. espanoli*, Arenales del Sol, Alicante (g), and spermatogonial mitotic chromosomes of *T. espanoli* (f), all showing a single bivalent or single pair of autosomes labelled. Fluorescent DAPI staining of meiotic metaphase I in *T. perezi* (d) and *T. espanoli* (h) shows a more intense signal in the chromatin with the ribosomal genes (indicated by arrows); in the latter plate other DAPI-positive signals appear associated to possibly AT-rich telomeric areas of rod-shaped bivalents not bearing ribosomal genes. Scale bar = 5 μm

the formerly supposed predominant mechanism of karyotype change, believed to be an increase in chromosome number over time (Petitpierre 1970b, 1973). However, former claims were not based on a phylogeny, which provides a framework for the assessment of polarity in character change. On the other hand, errors in the estimation of the phylogeny will have misleading effects on its use for further analyses of character evolution. But to date, the phylogenetic anchor used in this study is the most robust and information-rich evolutionary hypothesis available for *Timarcha* (Gómez-Zurita 2004).

Ignoring the quantitative implications of the previously discussed tests, it is rather apparent that the trend or at least the higher occurrence of fusion events leading to reduction in chromosome number is a plausible interpretation of the data. The most parsimonious reconstruction of character states throughout the phylogeny allows distinguishing only four unambiguous changes in chromosome number (Fig. 6). Of these, three are decreases from $2n = 20$ to $2n = 18$ in *T. aurichalcea*, from $2n = 28$ to $2n = 20$ in *T. marginicollis*, and from $2n = 28$ to $2n = 26$ in the lineage of *T. rugosa*; the

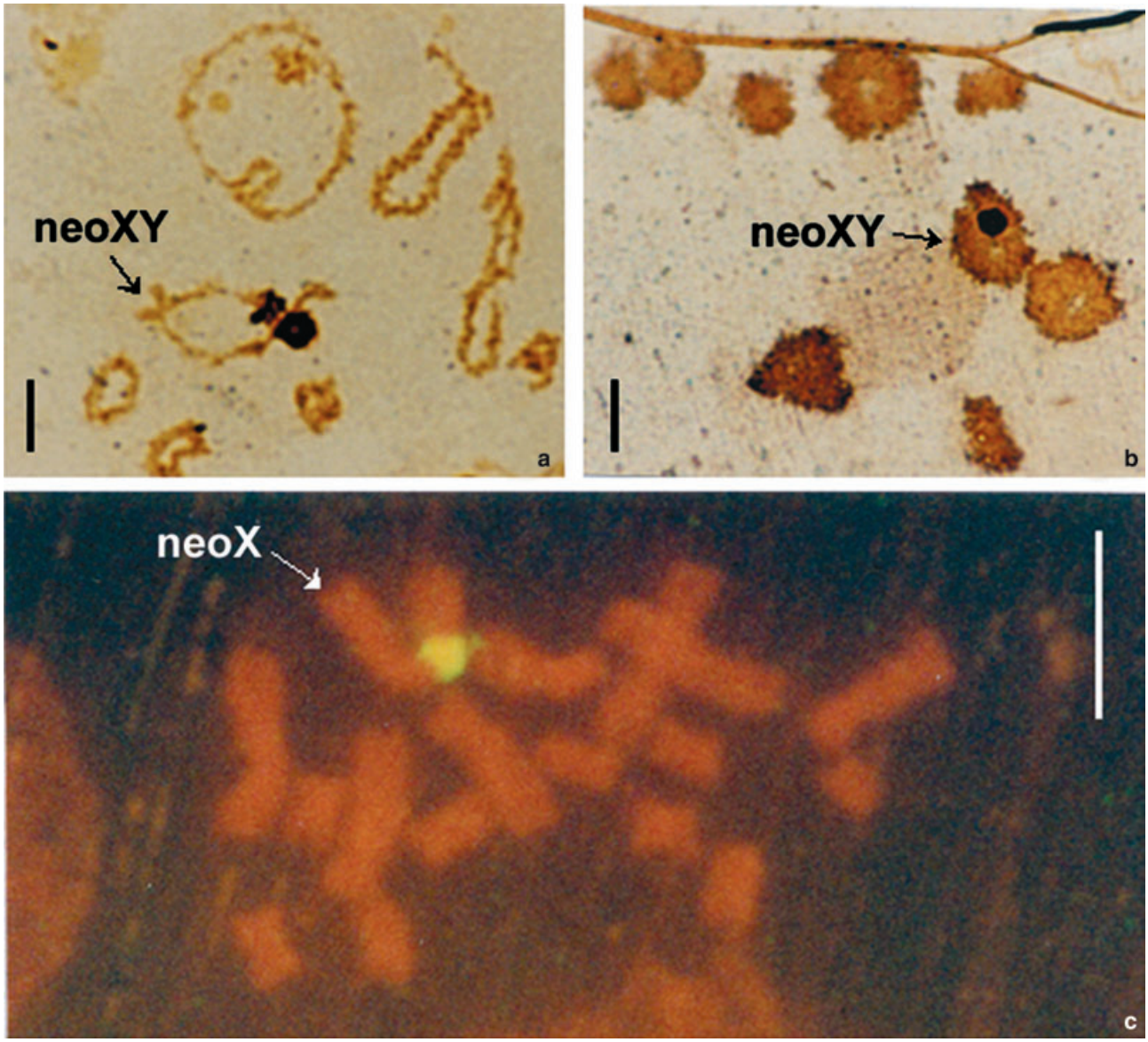

Fig. 5. Silver staining of meiotic diplotene (a) and metaphase I (b) divisions of *Timarcha aurichalcea*, Tragacete, Cuenca. The silver precipitate appears associated to the NOR-bearing chiasmatic and asymmetric sexual bivalent in both cases. Localization of the rDNA gene cluster in spermatogonial mitotic chromosomes of *T. aurichalcea*, exclusively on the pericentromeric area of the largest chromosome, identified as the neoX (c). Scale bar = 5 μm

only unambiguous increase is from $2n = 20$ to $2n = 22$ in *T. granadensis*.

Evolutionary origin of the *T. aurichalcea* karyotype

The species *T. aurichalcea* shows the lowest chromosome number reported so far for the genus, with $2n = 18$, and also shows the unique instance in *Timarcha* of a chiasmatic sexual chromosome pairing, instead of the most common 'parachute' association typical for this beetle family and for the Coleoptera at large (Smith and Virkki 1978). The availability of an independent phylogenetic hypothesis for *Timarcha*, tightly relating *T. aurichalcea* to *T. fallax* and the group of *T. perezi*, offers an excellent opportunity to investigate the evolutionary origin of the unusual karyotype of this taxon.

The karyotype of *T. aurichalcea* appears in the phylogeny as a recent derived condition from the widespread karyotype characteristic of the *T. goettingensis* species complex (Fig. 6), typically showing three pairs of large meta- or submetacentric autosomes, a large X-chromosome, a small-medium pair of acrocentric chromosomes, five pairs of small metacentric chromosomes and a minute Y chromosome (Petitpierre 1970b, 1976). A first examination of both karyotypes (see Fig. 1c–d), immediately reveals that *T. aurichalcea* lacks the pair of acrocentric chromosomes and that the Y chromosome is significantly larger than any of the small autosomes or the homologue chromosome in other species of the complex. Could the reduction in chromosome number in this species be the result of a fusion involving the fourth pair of chromosomes and the sexual chromosomes? We believe that the answer is positive based on the evidence obtained from Ag-NOR

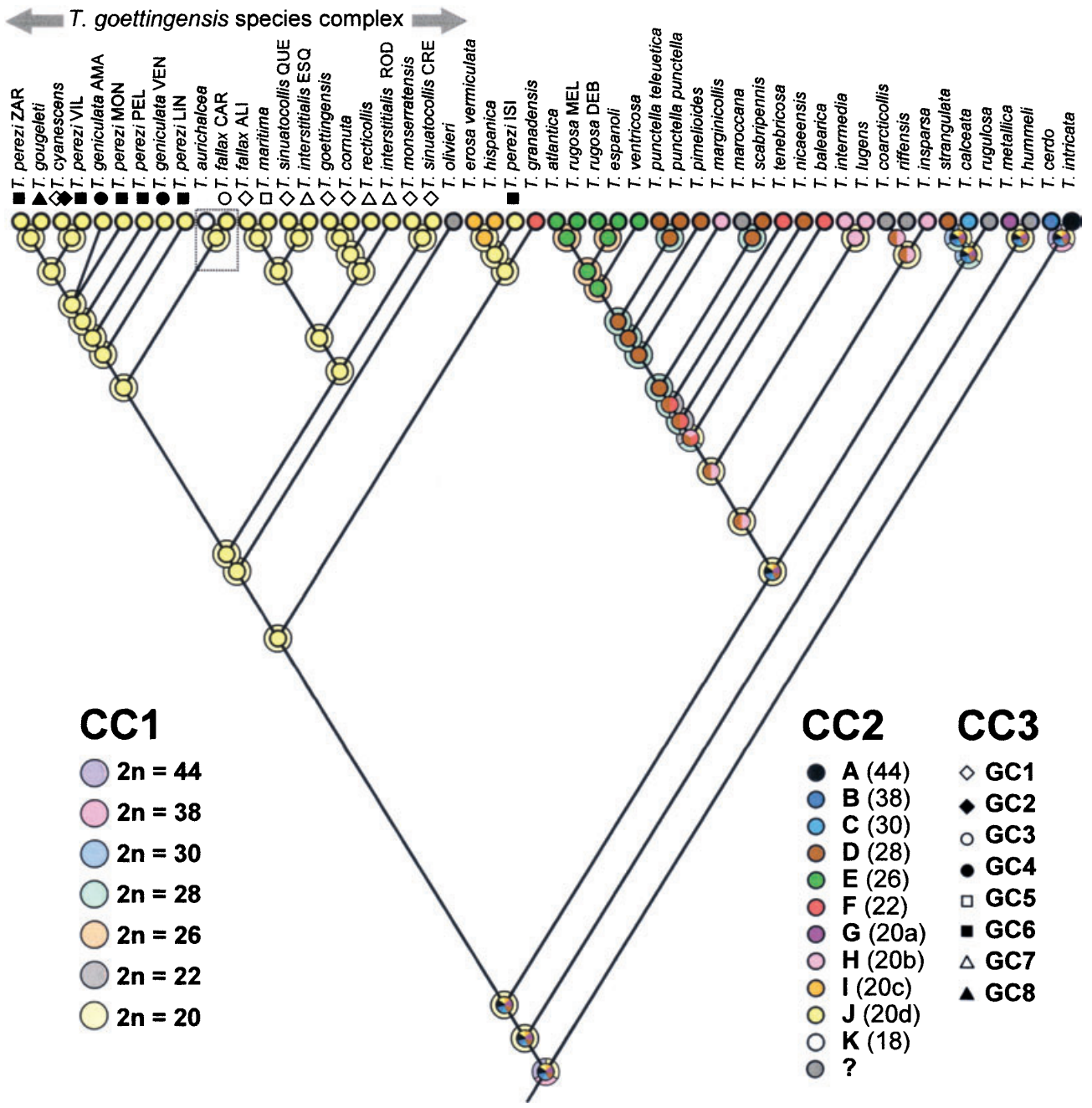

Fig. 6. Character optimization on the independent *Timarcha* phylogeny of Gómez-Zurita 2004 of the number of chromosomes (CC1) and further subdivision of the $2n = 20$ class in four other categories (CC2): G (20a: karyotype of the ancestral *T. metallica*), H (20b: regular size-decreasing karyotype), I (20c: karyotype with two large pairs of autosomes), and J (20d: *T. goettingensis* complex karyotype, with three large pairs of autosomes and a characteristic fourth pair of acrocentric chromosomes). Character distribution for a subdivision of the J class according to positional information of the centromere in several chromosomes is also shown (CC3). The three-letter code used for taxa represented more than once in the phylogeny refers to their specific sampling localities (for details see Gómez-Zurita 2004). The dotted box indicates the lineage with chromosome reduction due to centric fusions

banding and FISH analysis of this species and its close relatives.

The ribosomal gene cluster in *T. perezi* and *T. fallax*, and presumably in all the other species in the *T. goettingensis* complex, is localized in the short satellite arm of the acrocentric fourth pair of autosomes. Moreover, the localization of NORs in satellite acrocentric chromosomes is very commonly found in animals (Sumner 2003). Therefore, this specific marker could trace rearrangements involving this chromosome. In particular, in *T. aurichalcea* both the Ag-NOR staining and FISH signals of the ribosomal probe appear associated to the asymmetric sexual bivalent and to the X-chromosome, respectively. We have hypothesized two reciprocal centric fusions of the two acrocentric autosomes with each of the sexual chromosomes, reducing the chromosome number from 20 to 18 (Fig. 7). These fusions would have resulted in the loss of two centromeres, from the X chromosome in the X:4 fusion, resulting in a neoX chromosome

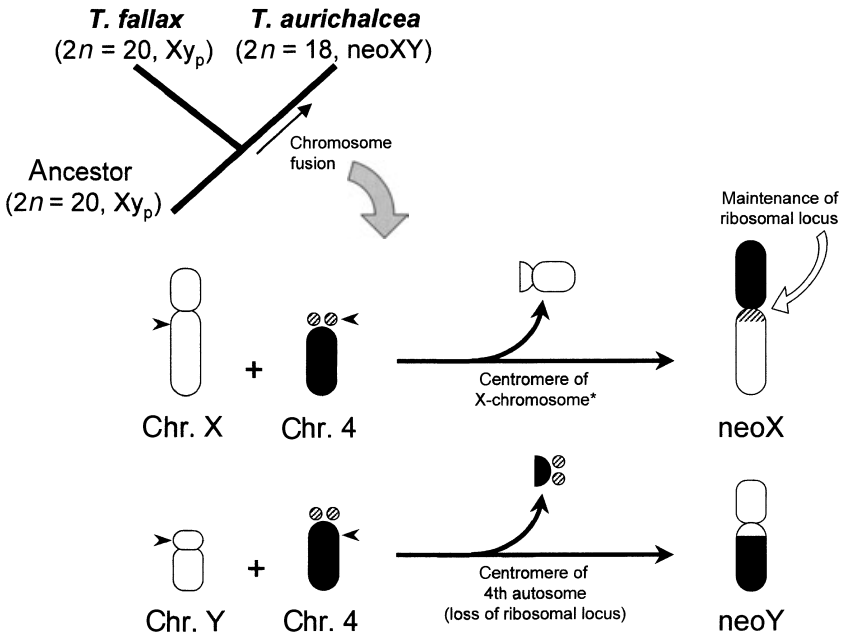

Fig. 7. Schematic interpretation of the hypothesized chromosome rearrangements occurred in the origin of the novel karyotype of *T. aurichalcea* ($2n = 18$, neoXY) from the ancestral *T. goettingensis*-complex karyotype ($2n = 20$, Xy_p). A reciprocal chromosome fusion between each of the sexual chromosomes with the NOR-bearing acrocentric fourth chromosome of the ancestor karyotype produces two new larger sexual chromosomes. The neoX chromosome conserves the rDNA loci contributed by the fourth autosome, while in neoY formation it is lost the rDNA satellite arm of the autosome, explaining that the rearranged chromosome lacks its signal. An asterisk denotes that the lost of the X centromere is not necessarily accompanied by loss of chromatin providing that the rearranged ancestral X-chromosome was acrocentric

bearing the ribosomal gene cluster associated near to the autosomal centromere, and loss of the autosomal centromere in the Y:4 fusion, with the consequent loss of the satellite short-arm including the ribosomal gene cluster. The alternative possibility is not viable because females would lack the essential ribosomal genes, while the option of both sexual chromosomes carrying ribosomal genes simply seems unlikely to have happened. Our interpretation of the chromosome rearrangements implies that there has been some loss of chromatin as well, particularly in the case of the original X-chromosome. However this is not necessarily the case, because the ancestor that underwent the rearrangement could have had an acrocentric X-chromosome as observed in some extant populations of the closely allied *T. perezi* (Petitpierre 1970b). In the scenario described here, the homologous portion of the fourth chromosome forming part of the neoX and neoY chromosomes in *T. aurichalcea* would be responsible for the chiasmatic pairing of the sexual bivalent observed in meiosis (Fig. 5a).

The episodic karyotype differentiation in this species, geographically restricted to small mountainous parts of central eastern Iberia, could have established reproductive barriers with other presently parapatric or allopatric populations/species, resulting in speciation for this taxon.

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Zusammenfassung

Die evolutive Entstehung eines neuen Karyotyps bei Timarcha (Coleoptera, Chrysomelidae) und der allgemeine Trend der Chromosomenevolution in dieser Gattung

In dieser Arbeit werden die Karyotypen von sechs Arten der Gattung Timarcha das erste Mal dargestellt und die zytologischen Beschreibungen für zwei weitere Taxa überprüft; in einem Fall (*T. erosa vermiculatra*) kann die frühere Beobachtung bestätigt werden, im anderen Fall (*T. scabripennis*) aber nicht. Wir beschreiben den beachtenswerten Karyotyp von *T. aurichalcea*, der die geringste Chromosomenzahl der Gattung ($2n = 18$) aufweist und der außerdem durch das Auftreten von ungewöhnlichen chiasmatischen Geschlechtsbivalenten, wie sie bisher bei Timarcha nicht beschrieben wurden, auffällt. Die vorliegende Untersuchung erhöht die Zahl der zytologisch untersuchten Arten dieser Gattung auf vierzig. Außerdem wurden zytologische Analysen, die Ag-NOR-Färbung und FISH-Studien mit rRNA-Proben umfassen, an mehreren Arten durchgeführt. Die Evolution des Karyotyps wird mittels verschiedener, den Karyotyp bestimmender Gesetzmäßigkeiten, die von einer publizierten unabhängigen phylogenetischen Hypothese für Timarcha aus einer Untersuchung von drei genetischen Markern kommt, abgeleitet. Die Anwendung eines Maximum-Likelihood-Modells mit optimierten Merkmalsveränderungen im Ablauf der Phylogenie wird schrittweise eingesetzt, um die möglichen Verschiebungen bei den Chromosomenveränderungen zu erkennen. Die Analysen zeigen, daßder Karyotyp in der Evolution dieser Gattung konservativ ist, daß aber ein auffälliger Trend zur Veringerung der Chromosomenzahlen besteht. Cytologische und phylogenetische Daten werden benutzt, um die evolutive Entstehung des Karyotyps von *T. aurichalcea* durch zwei zentrische Fusionen, bei denen ein akrozentrisches Autosomenpaar und die Geschlechtschromosomen beteiligt sind, zu erklären.

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