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Cytological and molecular differences in the *Ceroglossus chilensis* species complex (Coleoptera: Carabidae)

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Three sympatric forms have been found within the morphospecies *Ceroglossus chilensis* Esch. through karyotype analysis and characterization of repetitive DNA families. The male chromosome number of these forms is 2n = 28 + XY (form A), 2n = 40 + XY (form B), and 2n = 38 + trivalent (form C). Restriction enzyme analysis of total genomic DNA of form B using EcoRI produces several conspicuous bands corresponding to monomers and dimers of two repetitive DNA families, of about 575 bp and 200 bp respectively; form C has, after total digestion, only one band of 625 bp corresponding to the monomer of a different repetitive DNA family; and form A has no conspicuous band after this treatment. *In situ* hybridization experiments show that repetitive DNAs are specific for each form and no crossed hybridization is observed. The results suggest that these *Ceroglossus* forms are actually a complex of sibling species evolving as separate evolutionary and phylogenetic units, whose characterization at the morphological, ecological and geographical levels should be the subject of further investigation.

1. Introduction

According to Mayr (1969), "sibling species are pairs or groups of closely related species which are reproductively isolated but morphologically identical or nearly so". Reports on sibling species are not very frequent among carabid beetles, although there are many potential cases within the large amount of varieties and subspecies described for many species of this large family. Sibling species can be identified according to different types of characters: subtle morphological differences (Koch & Thiele 1980, Aukema 1990), chromosomes (Nettmann 1976, Serrano 1980a, 1982, Maddison 1985), electrophoresis of allozymes (Jermiin *et al.* 1991) or mitochondrial and nuclear DNA sequences (Vogler & DeSalle 1993, 1994ab). Some of these suggested species have been confirmed to be reproductively isolated, in agreement with Mayr's criteria (Koch & Thiele 1980, Aukema 1990).

Ceroglossus chilensis is interesting to us for two reasons: (1) it includes twelve sympatric or allopatric varieties and subspecies (Breuning 1928), and (2) it is one of the few members of the small tribe Ceroglos-

sini. The first point indicates a high degree of variation that deserves investigation. On the other hand, we are interested in analysing the karyotypic and molecular evolution of the whole supertribe Carabitae, to which Ceroglossini belongs. The hypothesis to be tested is that *Ceroglossus chilensis* is a single species and thus, significant differences between populations at chromosomal and molecular levels are unexpected.

2. Material and methods

2.1. Material

Twenty two individuals of *C. chilensis* were collected in Chile from natural populations in four localities (Table 1) . Identification was made by two of the authors (F. P. and D. M.) following Breuning (1928) and all the individuals studied were morphologically attributable to *C. chilensis* Eschscholtz.

2.2. Chromosome preparations

Karyological analyses were carried out on gonads dissected from beetles anaesthetized with ethyl-acetate. In some cases a sodium acetate plus 0.05% colchicine solution was injected 1 h prior to dissection. The gonads were fixed in fresh ethanol-acetic acid solution (3:1) for 1 h. Small sections of the gonads (1 mm) were squashed in 45% acetic acid and the coverslip removed after freezing in liquid nitrogen. Occasionally, small sections of gonadal tissue were placed in 20 µl of 60% acetic acid to break up the tissue in order to have a cell suspension. Drops of this suspension (5 µl) were placed on preheated slides and dried on a 60˚C hot plate. Dried cell preparations were stained with 3% Giemsa in phosphate buffer, pH 6.8. At least five cells per individual were analysed.

2.3. DNA extraction and endonuclease digestion

Genomic DNA was extracted using standard protocols. Whole individuals were homogenizated in a buffer (20mM Tris, 10 mM EDTA, 0.5% SDS) containing proteinase K and the mixture was incubated for 60 min at 65˚C. The homogenate was pelleted by centrifugation at 2 000*g* and then extracted with phenol:chloroform:isoamylalcohol (25:24:1). The aqueous phase was precipitated with 3 M sodium acetate and cold ethanol. Endonucleases assayed were EcoRI, DraI, TaqI, ClaI, HindIII, HaeI, SstI, HpaI, and MspI (Promega and Pharmacia). Endonuclease digestions were made following standard protocols (Sambrook *et al*. 1989). Briefly, total DNA was incubated with one or two units of the enzyme in the corresponding buffer for 30–60 min at 37˚C. Digested DNA was separated on a 2% agarose gel and stained with ethidium bromide.

2.4. DNA probes

The monomeric bands of 625 bp, 575 bp and 200 bp observed in the digestion (see results) were extracted from the gel using a USBioclean MP kit (USB). The 625 bp and 200 bp bands were cloned into the pUC19 vector (Boehringer Mannheim) and labelled with biotin-16-dUTP (Boehringer Mannheim) using a nick translation kit (Sigma) and both the vector and the insert were used for *in situ* hybridization. The 575 bp band was directly labelled after extraction from the gel.

2.5. *In situ* hybridization

Chromosome spreads were pre-treated with DNase-free RNase in 2 × SSC for 1 h at 37˚C, followed by treatment with 0.005% pepsin in 10 mM HCl for 10 min, dehydration in a graded ethanol series, and air drying. The hybridization mixture containing 50% deionized formamide, 2 × SSC, 50 mM sodium phosphate, pH= 7.0, 10% dextran sulphate and 4 ng/µl of labelled probe was denatured at 70˚C for 5 min and placed on ice. The slides were denatured in a 70˚C hot plate for 2 min. A 30 µl aliquot of the denatured hybridization mixture was placed onto the slides, covered with a 20 × 20 mm coverslip, sealed with glue, kept in the plate at 70˚C for another 3 min and then transferred to a humid chamber at 37˚C for hybridization overnight. After hybridization coverslips were carefully removed and the slides were then given a stringent wash for 3–5 min in 50% formamide, 2 × SSC at 37˚C. Sites of probe hybridization were detected with avidin-fluorescein isotyocyanate (FITC, Vector Laboratories). The signal was amplified once (pCc625, and pCc200) or twice (pCc575) using goat anti-avidin-biotin (Vector Laboratories). Slides were counterstained with propidium iodide and mounted with antifade solution to prevent the fluorescence to fade away. Slides were examined with a Leitz photomicroscope and photographed with Kodak Ektachrome.

3. Results

3.1. Karyotype analysis

The chromosome number of the individuals analysed can be grouped in three karyomorphs, named A, B and C (Table 1).

Form A. 2n = 30, n = 14 + XY (male); 2n = 30 (female). One male and one female were found to belong to form A. Spermatogonial mitoses show 2n = 30 chromosomes. The karyogram arranged according to size and morphology of the chromosomes

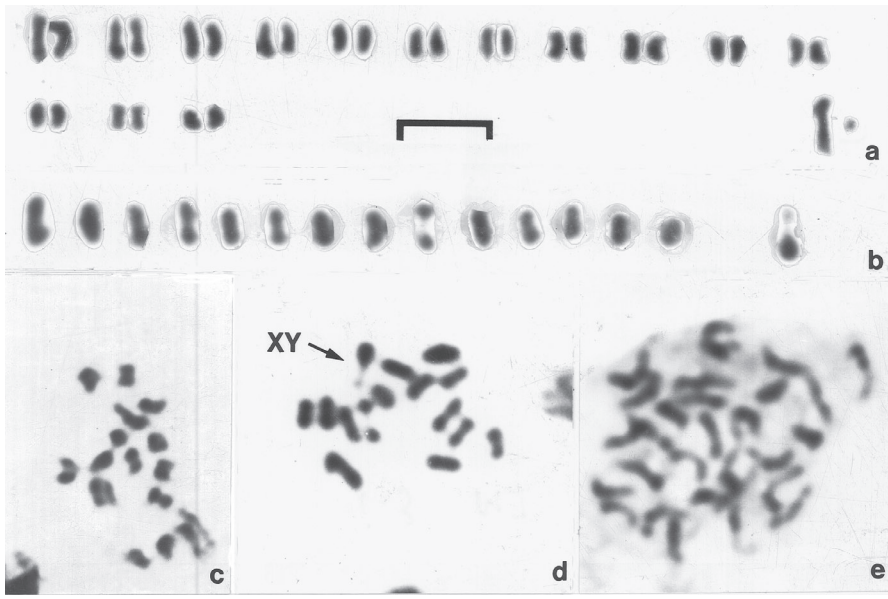

Fig. 1. Giemsa stained chromosomes of *Ceroglossus chilensis* form A. — a: male karyogram, 2n = 28 + XY, — b: male meiogram, n = 14 + XY, — c: male second metaphase plate with n = 14 + X, — d: male first metaphase plate with n = 14 + XY, — e: female mitotic premetaphase, 2n = 30. The sex chromosomes are figured to the right. Bar = 5 μm.

shows fourteen pairs of gradually decreasing size and two odd chromosomes, the largest and the smallest of the karyotype, that are identified as the sex chromosomes (Fig. 1a). This identification is congruent with observations of metaphase I cells, in which there are fifteen bivalents and one of them is clearly heteromorphic (Figs. 1b and d). Metaphase II cells show the expected fifteen chromosomes (Fig. 1c). Meiotic pairing of bivalents is regular and one chiasma per bivalent is the rule. In females only premetaphase mitotic stages with 30 chromosomes were observed (Fig. 1e).

Form B. 2n = 42, n = 20 + XY (male); 2n = 42 (female). Male karyograms show 21 pairs of chromosomes with a heteromorphic pair made up by the largest and the smallest members, that probably corresponds to the XY pair as in form A (Fig. 2a). The same figure is observed in female karyograms where the largest chromosome observed in males is in duplicate, corresponding to the XX pair (Fig. 2b). The identification of the sex chromosomes is corroborated by the presence of a heteromorphic bivalent in male meiotic cells (Fig. 2c). Meiotic pairing is regular with one terminal or subterminal chiasma per bivalent. Cells with n = 20 + X (Fig. 2d) and others with n = 20 + Y are observed in metaphase II (Fig. 2e).

Form C. 2n = 41 + Bs, n = 19 + III + Bs (male); 2n = 42? (female). This form is related to form B but can be differentiated from it at least by a lower male chromosome number and the presence of a trivalent in meiosis. Spermatogonial metaphase plates show 41 chromosomes (Fig. 3a) although some individuals have two additional chromosomes which

Table. 1. Chromosome number, type of repetitive DNA and collecting localities of the individual of *Ceroglossus chilensis* studied.

Form	2n	n	Repetitive DNA cut with EcoRI	No. of indiv.	Loc.
A	30♂/30♀	14 + XY	–	1♂,1♀	1
B	42♂/42♀	20 + XY	pCc575 pCc200	3♂♂,2♀♀	1, 2
C	41 + Bs♂/ 42?♀	19 + III + Bs	pCc625	12♂♂,3♀♀	1, 2, 3, 4

1. Pedernales. Estación Concordia. Provincia de Llanquihue, 2. Ñancul. Provincia de Valdivia, 3. Estación Victoria, 4. Estero Huachítico. Provincia de Cautín.

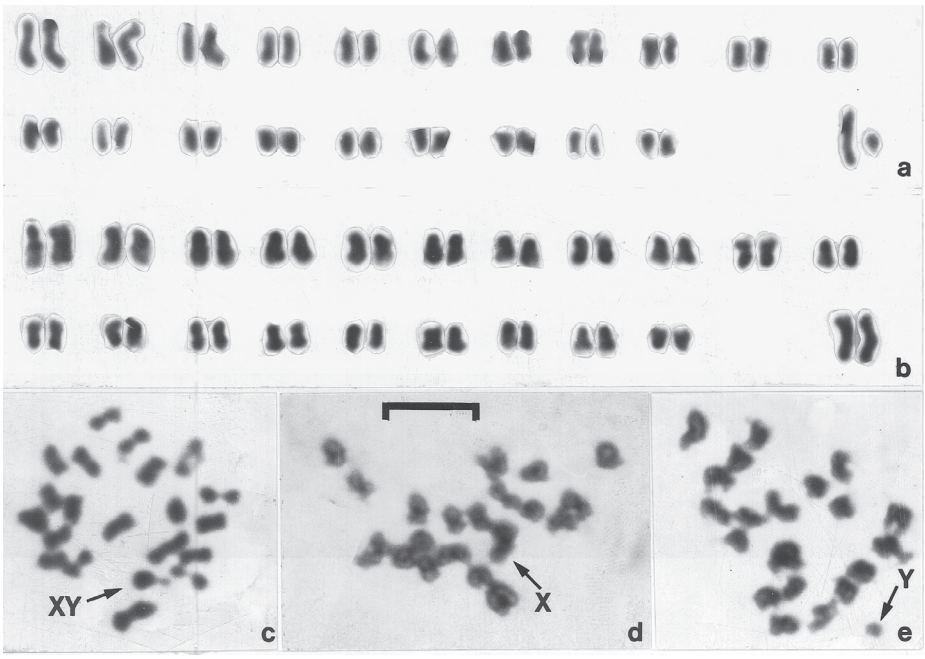

Fig. 2. Giemsa stained chromosomes of *Ceroglossus chilensis* form B. — a: male karyogram, 2n = 40 + XY, — b: female karyogram, 2n = 40 + XX, — c: male first metaphase plate with n = 20 + XY, — d and e: male second metaphase plates with n = 20 + X (d) and n = 20 + Y (e). The sex chromosomes (XY and XX) are figured to the right in a and b, respectively. Bar = 5 μm.

we think are B-chromosomes (Fig. 3b). There is a marked difference in size between the first and the second pair, both in mitotic and in meiotic chromosomes, which was not observed in the other two forms. Metaphase I cells show 19 bivalents plus a trivalent (20 pairs in cells with a supernumerary pair; Fig. 3c and d). In male meiosis the autosomes pair following a chiasmatic pattern in which there is one chiasma per bivalent distally located. However, subterminal chiasmata are found in some bivalents (e.g. in pairs 6 and 7; Fig. 3d). The three chromosomes involved in the trivalent are associated by two terminal chiasmata giving rise to a figure in which co-orientation is very regular (Figs. 3e and f). Most metaphase II plates have 20 (Fig. 5a) or 21 chromosomes (Fig. 5b), but cells with one B-chromosome are also present. Results on females are not conclusive as counts on ovogonial cells show varying numbers of chromosomes between 40 and 42 but they are expected to show 2n = 42 when better cells are analysed. Ordination of chromosomes in a karyogram (Fig. 3i) suggests that the second large pair corresponds to one of the odd elements that make the trivalent in males, so this may be tentatively identified as an X chromosome.

3.2. Detection of repetitive DNA

Form C. Digestion with EcoRI and DraI produced one conspicuous band of about 625 bp corresponding to satellite DNA (Fig. 4; Lane 4). Partial digestion of genomic DNA with EcoRI showed a series of bands arranged in a ladder corresponding to the expected monomers, dimers, trimers, etc., indicating a tandem disposition of the repetitive DNA in the genome. Single digestions with the endonucleases HpaI and MspI showed a pattern of fragments ranging from a few bp to about 380 bp. Double digestion with DraI and MspI did not show the conspicuous band expected after cutting with DraI, but showed the pattern observed after a single digestion with MspI, thus indicating that the 625 bp satellite DNA has several internal targets for MspI, so that, only one family of repetitive DNA was detected (Fig. 5). The other enzymes assayed did not

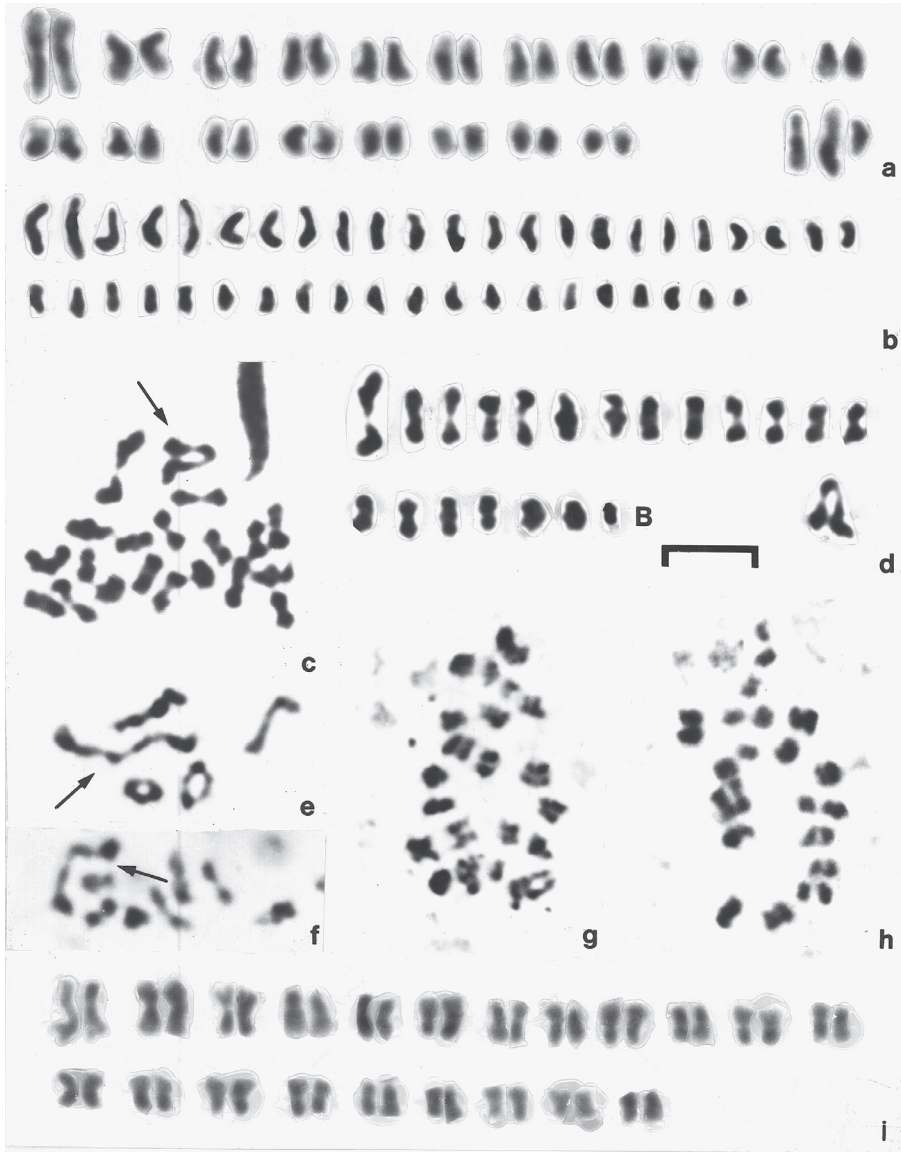

Fig. 3. Giemsa stained chromosomes of *Ceroglossus chilensis* form C. — a: male karyogram, 2n = 41; sex chromosomes tentatively identified are figured to the right, — b: male karyogram, 2n = 41 + 2Bs, arranged according to chromosome size, — c: male first metaphase plate, n = 19 + trivalent + Bs, — d: Meiogram of the same cell arranged according to decreasing size of bivalents, — e and f: Details of meiotic cells with the trivalent, — g: male second metaphase plate with n = 21, — h: male second metaphase plate with n = 20, — i: Female karyogram, 2n = 42. The trivalent in c, e and f is marked by arrows. Bar = 5 μm.

cut the repetitive DNA. According to these results, only EcoRI and DraI endonucleases were assayed in the other two chromosomal variants.

Form B. Individuals of chromosome form B showed two bands of about 575 bp and 200 bp after total digestion with EcoRI (Fig. 4; Lane 6). Partial digestions showed a band between them of about 400 bp, corresponding to the dimer of the 200 bp band. Total digestion with DraI showed a band of about 575 bp, just the size of the large band resulting

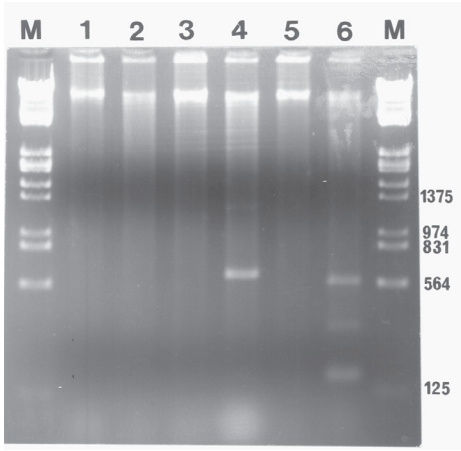

Fig. 4. Agarose gel electrophoresis of total DNA of Ceroglossus chilensis forms A, B and C. — Lane 1: undigested total DNA of form A. — Lane 2: DNA of form A after digestion with EcoRI; note the lack of bands. — Lane 3: Undigested total DNA of form C. — Lane 4: DNA of form C after digestion with EcoRI; note the presence of a 625 bp band (monomer) and a 1,250 bp band (dimer). — Lane 5: Undigested total DNA of form B. — Lane 6: DNA of form B after digestion with EcoRI; note the presence of a 200 bp band (monomer) and a 400 bp band (dimer) of a repetitive DNA family and a band larger than 575 bp corresponding to the monomer of another repetitive DNA family. Lanes M correspond to phage DNA digested with EcoRI and HindIII used as size marker.

after digestion with EcoRI, but not the small one of about 200 bp. This suggests that form B has at least two repetitive DNA families with monomers of about 575 bp and 200 bp respectively.

Form A. DNA from individuals of chromosome form A did not show any band after total digestion with EcoRI (Fig. 4; Lane 2) and DraI, thus indicating that the repetitive DNA of this form, if present, has a different sequence from those ones of forms C and B.

The single band of form C (625 bp) and the small band (200 bp) of form B were cut from the gel, and thereafter the DNA was purified, cloned and labelled with biotin. The probes are referred to as pCc625 and pCc200 respectively. The large band (575 bp) of form B was cut from the gel, and the DNA was purified and directly labelled with biotin. This probe is referred to as pCc575. The three probes were used for fluorescence *in situ* hybridization experiments.

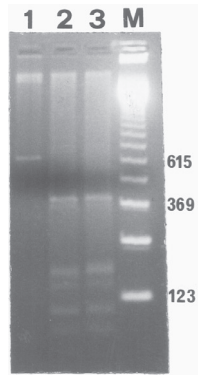

Fig. 5. Agarose gel electrophoresis of total DNA of *Ceroglossus chilensis* form C after endonuclease digestion. — Lane 1: DNA digested with DraI. — Lane 2: DNA digested with MspI. — Lane 3: double digestion of DNA with DraI and MspI. — Lane M corresponds to 123 bp ladder used as size marker.

3.3. *In situ* hybridization

Probes pCc625, pCc575 and pCc200 were used for fluorescent *in situ* hybridization on nuclei of the three chromosomal forms. Sites of hybridization were detected as yellow fluorescent dots on the red nuclei counterstained with propidium iodide. No cross-hybridization was observed in the experiments. Nuclei of individuals belonging to form A did not show any hybridization signal with the three probes obtained from the other two forms. Nuclei of individuals belonging to form B show hybridization using probes pCc575 and pCc200 obtained from other individuals of the same form, but did not show any signal using probe pCc625 obtained from individuals of form C. Nuclei of individuals belonging to form C showed hybridization using probe pCc625 obtained from individuals of the same form, but did not show any signal after hybridization with probes pCc575 and pCc200 obtained from individuals of form B. The nucleotide sequence and chromosomal localization of these repetitive DNA families will be presented elsewhere (Galián *et al*. in prep.)

4. Discussion

4.1. Chromosome number

The chromosome number observed in 19 of the 22 individuals used in the study indicates that there are three well differentiated karyological forms. Individuals of form A, with n = 14 + XY (males), have a karyotype very similar to that with n = 13 + XY present in *Carabus* and *Calosoma* species (Serrano 1980b, 1981, 1986, Serrano & Yadav 1984, Galián *et al*. 1992) in terms of morphology and relative size

of autosomes, sex chromosome system and morphology and relative size of heterosomes. The main difference is due to the presence of an additional pair of autosomes in form A of *C. chilensis*. Therefore, we suggest that this form is close to the ancestral condition of the genus, whereas forms B and C are derived states originated through repeated changes that increased the number of chromosomes. *C. chilensis* form B, with n = 20 + XY (males), has a karyotype with six chromosome pairs more than form A, but with similarities in the morphology of the largest autosomal pairs, sex chromosome system and morphology and relative size of the heterosomes. Arrangement of the female karyotype corroborates the tentative ordination of males, showing the X chromosome in duplicate. *C. chilensis* form C, with n = 19 + III (males) is related to form B with regard to the high chromosome number, but has one chromosome less and shows a trivalent in male meiosis. Moreover, the first autosomal pair is better recognised in this form than in form B because of its larger size with regard to the next pair. The trivalent may correspond to an XXY sex chromosome system originated through an X-autosome fusion in a form with the 42 chromosomes and the usual XY system found in form B. This interpretation of the trivalent is based upon the chromosome number observed in both sexes of form C and the lack of the typical heteromorphic XY pair found in other XY species, such as forms A and B, and species of the genera *Carabus* and *Calosoma*.

4.2. Differences in repetitive DNA

Forms B and C have different repetitive DNA families whereas form A has not shown any band with the endonuclease EcoRI. The probe obtained after cloning the 625 bp band of form C (pCc625) did not show a fluorescent signal after hybridization on nuclei of forms A and B, thus indicating either that the nucleotide sequence of the repetitive monomer of form C is not present in the other two forms, or that it is present in such low copy numbers that it can not be detected from the background. The same holds for the probes obtained from form B.

The fact that these three forms have accumulated simultaneously differences in the number of chromosomes and the repetitive DNA, suggests that one of the factors that have made possible the increase in the number of chromosomes is the accumulation of repetitive DNA in some lineages of *Ceroglossus*. This hypothesis is congruent with the fact that species of the related genera *Carabus* and *Calosoma*, do not show repetitive DNA after digestion with EcoRI and DraI (Galián, unpubl. data), the same result that has been obtained in form A, which is the more akin to these genera in terms of chromosome number.

4.3. Evolutionary and phylogenetic implications

Many different subspecies and varieties have been described under the morphospecies *C. chilensis*, even from the same locality (Breuning 1928). This suggests that we are dealing either with a complex of sibling species, or with a single species showing a remarkable level of polymorphism. The results reported here favours the first hypothesis, that is, the three forms are actually separate evolutionary units that would meet the criteria used for defining different types of species (biological, evolutionary, phylogenetic, etc.).

First, the three lineages differ from one another by the chromosome number, the sex chromosome system and the specific type of repetitive DNA. Form A would be more related to the ancestral species of the group according to the chromosome number, whereas forms B and C would be derived taxa, closely related to each other. Their karyotypic differences are hardly explained by some kind of intraspecific polymorphism. Remarkable cases of intraspecific numerical polymorphism are known for beetles of other genera such as *Chilocorus* (Smith & Virkki 1978) or for other animals such as the stick insect *Didymuria,* the grasshopper *Vandiemenella,* the isopod *Jaera,* and the rodents *Spalax* or *Mus* (White 1978). All of them seem to fit the stasipatric model put forward by White, in which chromosomal races have a parapatric distribution, a fact that allows for the preservation of the polymorphism. This is not the case for *C. chilensis*, because the three forms are found in sympatry (see Table 1).

Second, the results on repetitive DNA also correspond to differences between "good" species. According to Bachmann *et al.* (1991), "it is agreed that satellite (i.e. repetitive) DNA varies little between individuals of the same species, but can be drastically different between even closely

related species". Although there are some reports of closely related species with almost identical satellite DNA sequences (Lohe & Brutlag 1987), the cases in which differences in this kind of DNA are found correspond to species well identified by other characters, as it happens with the beetle genus *Tribolium* (family Tenebrionidae; Juan *et al*. 1993) or *Drosophila* (Bachmann *et al*. 1991). Thus, the case of *C. chilensis* would constitute one of the first reports of cryptic sibling species detected by differences in repetitive DNA families.

The hypothesis about the existence of, at least, three sibling species within the morphospecies *Ceroglossus chilensis*, deserves further corroboration from other characters, including detailed studies of morphology, ecology and distribution, in order to understand the evolutionary picture of this group of beetles and improve accordingly its taxonomy and phylogeny.

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