The localization of mitochondrial sequences to chromosomal DNA in orthopterans

H.E. Vaughan, J.S. Heslop-Harrison, and G.M. Hewitt

Abstract: There is growing evidence that the integration of mitochondrial DNA sequences into nuclear and chloroplast genomes of higher organisms may be widespread rather than exceptional. We report the localization of 18S–25S rDNA and mitochondrial DNA sequences to meiotic chromosomes of several orthopteran species using in situ hybridisation. The cytochrome oxidase I (COI) sequence localizes to the centromeric and two telomeric regions of the eight bivalents of *Chorthippus parallelus*, the telomeric regions in *Schistocerca gregaria* and is present throughout the genome of *Italopodisma* sp. (Orthoptera: Acrididae). The control region of the mitochondrion and COI localize to similar chromosomal regions in *S. gregaria*. These data explain sequencing data that are inconsistent with the COI sequence being solely mitochondrial. The different nuclear locations of mtDNA in the different genera studied suggest that grasshopper mtDNA-like sequences have been inserted into the nuclear genome more than once in Acridid history, and there may have been different mechanisms involved when these events occurred in each of these species.

Key words: *Schistocerca gregaria*, *Italopodisma* spp., *Chorthippus parallelus*, in situ hybridisation, mitochondrial DNA, genome organization.

Résumé : Il y a des indications croissantes suggérant que l'intégration de séquences d'ADN mitochondrial au sein des génomes nucléaire ou chloroplastique des organismes supérieurs pourrait être davantage la règle que l'exception. Les auteurs rapportent la localisation d'ADNr 18S–25S et d'ADN mitochondrial sur des chromosomes méiotiques chez plusieurs espèces d'orthoptères au moyen de l'hybridation *in situ*. La séquence codant pour la cytochrome oxydase I (COI) est présente dans une région centromérique et deux régions télomériques parmi les huit bivalents du *Chorthippus parallelus*, dans des régions télomériques du *Schistocerca gregaria* et partout dans le génome de l'*Italopodisma* sp. (Orthoptera ; Acrididae). La région de contrôle mitochondrial et les séquences COI sont situées à des endroits semblables chez le *S. gregaria*. Ces données expliquent le fait que certains résultats de séquençage étaient incompatibles avec une origine uniquement mitochondriale du gène COI. Les différents emplacements nucléaires de ces ADNmt chez les différents genres étudiés suggèrent que ces séquences d'ADN d'origine mitochondriale ont été insérées dans le génome nucléaire à plus d'une reprise au cours de l'évolution des acridiens. De plus, différents mécanismes peuvent avoir conduit à ces événements chez les différentes espèces.

Mots clés : *Schistocerca gregaria*, *Italopodisma* spp., *Chorthippus parallelus*, hybridation in situ, ADN mitochondrial, organisation génomique.

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Introduction

Mitochondrial DNA has proved valuable in elucidating phylogenetic relationships, evolutionary pathways, and colonisation patterns (e.g., Talbot and Shields 1996; Verheyen et al. 1996; Thorpe et al. 1993; Juan et al. 1995, 1996). Its usually exclusive maternal inheritance has also allowed the elucidation of ancestral lineages and directions of hybridisations (Avise 1994).

Recent evidence, however, has shown that the use of mitochondrial sequences in phylogenetics can be misleading. Zardoya and Meyer (1996) point out the pitfalls of selecting mitochondrial sequences at random to construct phylogenies, and Zhang and Hewitt (1996) describe the ambiguities that can arise if more than one sequence is derived from each individual as a result of heteroplasmy or nuclear copies of the sequence under study. There are numerous studies, however, in which organellar sequences have been used apparently without problems, so it is difficult to know just how widespread is the occurrence of nuclear copies of organellar genes.

In fact, there are many documented cases of nuclear integration of chloroplast and mitochondrial sequences (Gellissen et al. 1983; Nugent and Palmer 1991), as well as transfer between these two types of organelle (Stern and Lonsdale 1982; Stern and Palmer 1984). It therefore seems timely and informative to attempt to localize visually such organellar

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sequences on the chromosomes and provide a method for assessing the extent of their occurrence more readily and generally.

In this study we have used a 1.2-kb probe from the cytochrome oxidase I (COI) gene sequence as a probe in situ to meiotic chromosomes of three grasshopper species: *Schistocerca gregaria*, *Italopodisma* sp., and two subspecies of *Chorthippus parallelus* (Orthoptera, Acrididae). We also used the 18S–25S nuclear ribosomal DNA repeat, and, in the case of *S. gregaria*, the AT-rich control region of the mitochondria, and the nuclear unique sequence *hox* as probes.

Materials and methods

Collection of grasshoppers

Individuals of *Chorthippus parallelus parallelus* (2*n* = 16 +XX/XO) were collected from French sites north of the Pyrenees. *Chorthippus parallelus erythropus* individuals (2*n* = 16 +XX/XO) were collected from Spain, south of the Pyrenees. Both collections were away from the hybrid zone (Hewitt 1993). *Italopodisma* sp. (2*n* = 22 + XX/XO) individuals were collected from Gran Sasso in Italy. *Schistocerca gregaria* (2*n* = 22 +XX/XO) individuals were bought from Blades Biological, Kent.

Preparation of slides for in situ hybridisation

Testes were dissected, fixed in a solution of 3:1 ethanol – glacial acetic acid at room temperature and subsequently stored at –20°C. Just prior to use, follicles were transferred to, and tapped out in, 45% acetic acid. Slides were viewed under phase contrast, dried at 37°C overnight and then desiccated at 4°C.

DNA extraction and probes

Bodies of grasshoppers were stored in 96% ethanol or frozen at –80°C. DNA extraction was modified from Sambrook et al. (1989). In the case of *S. gregaria*, separate mitochondrial and nuclear fractions of the genome were provided by Dr. D.-X. Zhang, prepared as described in Zhang et al. (1995).

PCR amplification was performed using Promega Taq polymerase. The cycle used was dependent on the primers employed for amplification in each case: (*i*) 1.2 kb of the COI gene amplified using primers UEA 3 (TATAGCATTCCCACGAATAAATAA) and UEA 10 (TCCAATGCACTAATCTGCCATATTA) (Lunt et al. 1996); (*ii*) approximately 1 kb of the AT-rich control region amplified using primers J1 (CGTATAACCGCGGCTGCTGGC) and J6 (GGTAATCCTTTAATCAGGCACTCC) (Zhang et al. 1995); (*iii*) a 295-bp region of *hox* using material and a protocol from D.-X. Zhang (Zhang and Hewitt, in preparation; see Zhang and Hewitt 1996).

The clone pTa71, containing the nuclear ribosomal DNA repeat unit with the 18S–5.8S–26S coding regions and intergenic spacers, cloned into the vector pUC19 from wheat was used as the rDNA probe (Gerlach and Bedbrook 1979).

Labeling and in situ hybridisation

Labeling of all probes was performed using digoxigenin–11-dUTP or biotin–16-dUTP by random priming. Efficiency of labeling was tested by dot-blotting (Sambrook et al. 1989).

In situ hybridisation was performed using techniques modified from Schwarzacher et al. (1989). All initial hybridisations were carried out in 40% formamide and 2× SSC (20× SSC: 3 M NaCl + 0.3 M sodium citrate) with approximately 50 ng of probe per slide; subsequent hybridisations were made by increasing the stringency through lowering the salt concentration to 0.75× SSC. Chromosome denaturation was performed at 60°C for 5 min for all species. Hybridisation was carried out at 37°C in a moist chamber overnight. After hybridisation, slides were washed in 20% formamide in 0.1× SSC at 42°C for 2× 5 min (the most stringent wash, enabling probe-target hybrids with more than 85% homology to remain stably hybridised), followed by washes in 2× SSC at 42°C for 2 × 5 min, 2× SSC at room temperature for 2× 5 min and 4× SSC plus 0.2% Tween–20 for 5 min.

Biotin-labeled probes were detected with Avidin-Cy3 (Sigma) at a dilution of 1:250, and digoxigenin probes were detected with anti-digoxigenin-FITC (Boehringer Mannheim) at a dilution of 1:75, according to the manufacturer's instructions. The preparations were treated with a 0.5 mg/mL solution of DAPI (4′,6-diamidino-2-phenylindole) and mounted in Citifluor (Agar Aids). Slides were examined by fluorescence microscopy, photographed on Fujicolor 400 Super HG colour print film, negatives scanned to PhotoCD (Kodak Digital Science), and processed using functions in Adobe Photoshop that affect the whole image equally. Most likely chromosome identities were assigned using chromosome size, morphology, and observed staining properties. Because we were using meiotic material, numerous (>30) cells were observed from each preparation, and several preparations from different animals were examined with each probe, including double-target hybridisations.

In situ hybridisation was performed with the COI gene from the homologous source in each case. As controls, double target in situ hybridisation was performed with an heterologous rDNA probe. Additional hybridisations were performed to chromosomes of *S. gregaria*. These included probing with a single copy sequence known to be nuclear (*hox*) and with the control region of the mitochondrion, both separately and together with the COI probe.

Results

Localization of rDNA

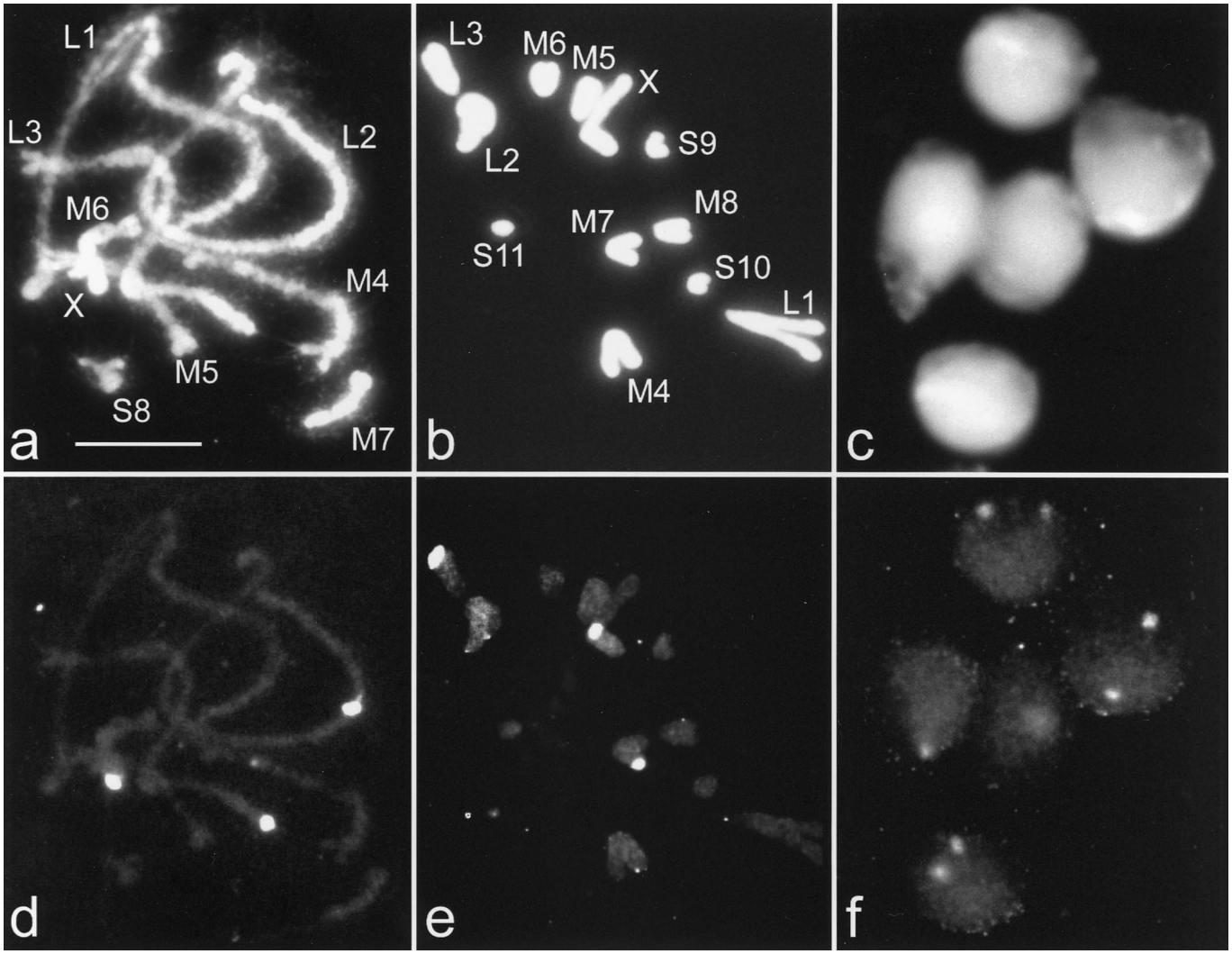
Ribosomal DNA localized to three major sites in *Chorthippus parallelus parallelus* (Figs. 1*a*, 1*d*) and two sites in *C.p. erythropus*. The sites were located proximally on the short arm of submetacentric chromosome L2 and distally on the short arm of submetacentric L3 in both subspecies, with the additional third site distal on the acrocentric X chromosome of *C.p. parallelus*. *Italopodisma* sp. (Figs. 1*b*, 1*e*) hybridised to sites pericentric on the X chromosome and distal on a large autosome, maybe L3, as well as a heterozygous third site, localized distally on a smaller autosome, perhaps M7. In *S. gregaria* (Figs. 1*c*, 1*f*) two NOR (nucleolar organizer region) sites were observed; these were localized interstitially on M6 and L3 (data not shown). No minor rDNA sites were observed in any species.

Localization of COI genes

In both subspecies of *Chorthippus parallelus*, centromeric regions of all eight bivalents and the X univalent were labeled with the COI mitochondrial sequence probe (Figs. 2*a*, 2*d*). There were also sites distally on the long arm of chromosomes L2 and both arms of M6.

In *S. gregaria* a single telomeric region was labeled in eight bivalents plus the X univalent (Figs. 2*b*, 2*e*). In two bivalents, the label was faint or absent at a sub-telomeric site. One large metacentric bivalent, probably L2, was always unlabeled. In zygotene and early pachytene nuclei, sites of hybridisation were often clustered. *Italopodisma* sp. showed label spread throughout the genome with some more weakly labeled chromosomes (Figs. 2*c*, 2*f*).

Fig. 1. Sites of in situ hybridisation of an 18S–25S ribosomal DNA probe (detected by a red fluorescent label; *d*, *e*, *f*) to orthopteran nuclei stained with DAPI (4′,6-diamidino-2-phenylindole, blue; *a*, *b*, *c*). Suggested chromosome numbers are indicated on the DAPI-staining micrographs; (*a*, *d*) *C.p. parallelus* pachytene nucleus showing three rDNA sites; (*b*, *e*) *Italopodisma* sp. half anaphase I showing three sites, one pericentromeric on the X chromosome and two located distally, on a large and medium autosome; (*c*, *f*) *S. gregaria* interphase nuclei showing two rDNA sites. Bar = 10 μm.



Localization of the AT-rich region and *hox* to *Schistocerca gregaria*

In situ hybridisation showed that the AT-rich mitochondrial control region localized to the same chromosomal segments as the COI probe in the *S. gregaria* genome (Fig. 3). Two of the three large bivalents from metacentric chromosome pairs were labeled sub-telomerically, while the third was unlabeled. Six of the medium and smaller bivalents, and the X chromosome, were labeled in the telomeric region, and two bivalents showed no or very faint labeling. Again, clustering of sites was seen in some nuclei.

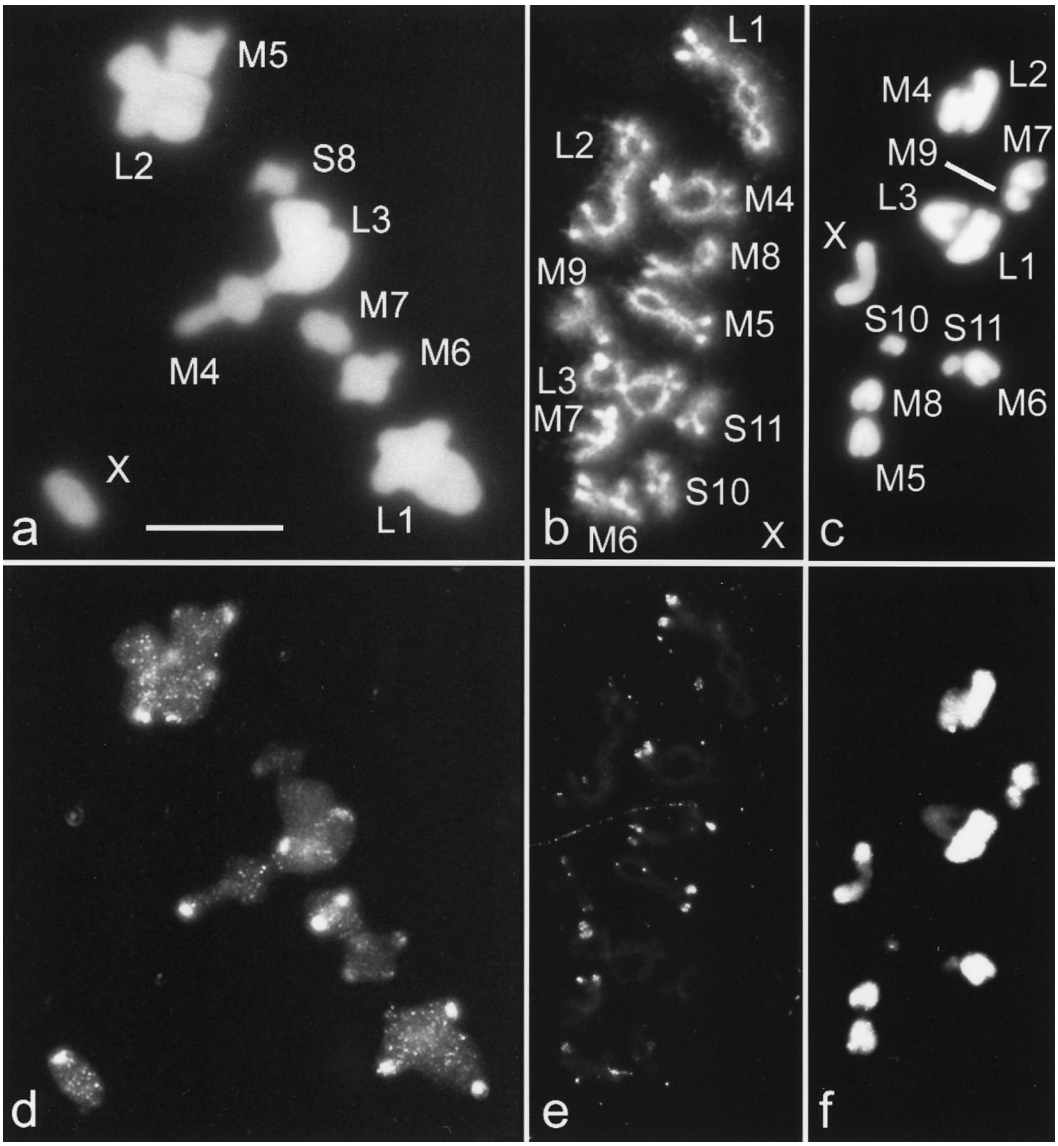
No labeling was detected when the *hox* sequence was used as a probe to chromosomes of *S. gregaria* (data not shown).

Discussion

The cytochrome oxidase I (COI) sequence and a probe for rDNA, were found to hybridise to chromosomes of the four taxa from three orthopteran genera that we investigated. A probe for the mitochondrial control region also hybridised to the chromosomes of *S. gregaria*. The patterns of hybridization observed did not correlate with known banding patterns for the chromosomes, while the rDNA hybridised specifically to known nucleolar organising chromosomes. We did not detect hybridization of *hox*, the single copy nuclear gene, suggesting that only multiple copies of a region will attract enough probe to be visible under the conditions employed here. The demonstration that mitochondrial sequences localize to different chromosomal locations in different species has implications for the organisation, evolution and plasticity of orthopteran genomes, as well as the use of organellar sequences in phylogenetic studies.

The localization of ribosomal DNA probes to meiotic preparations of *C. parallelus* chromosomes reveals a pattern of hybridisation that is consistent with previous silver-staining observations of active NORs (Gosalvez et al. 1988*a*). Two autosomal sites on chromosomes L2 and L3 are present

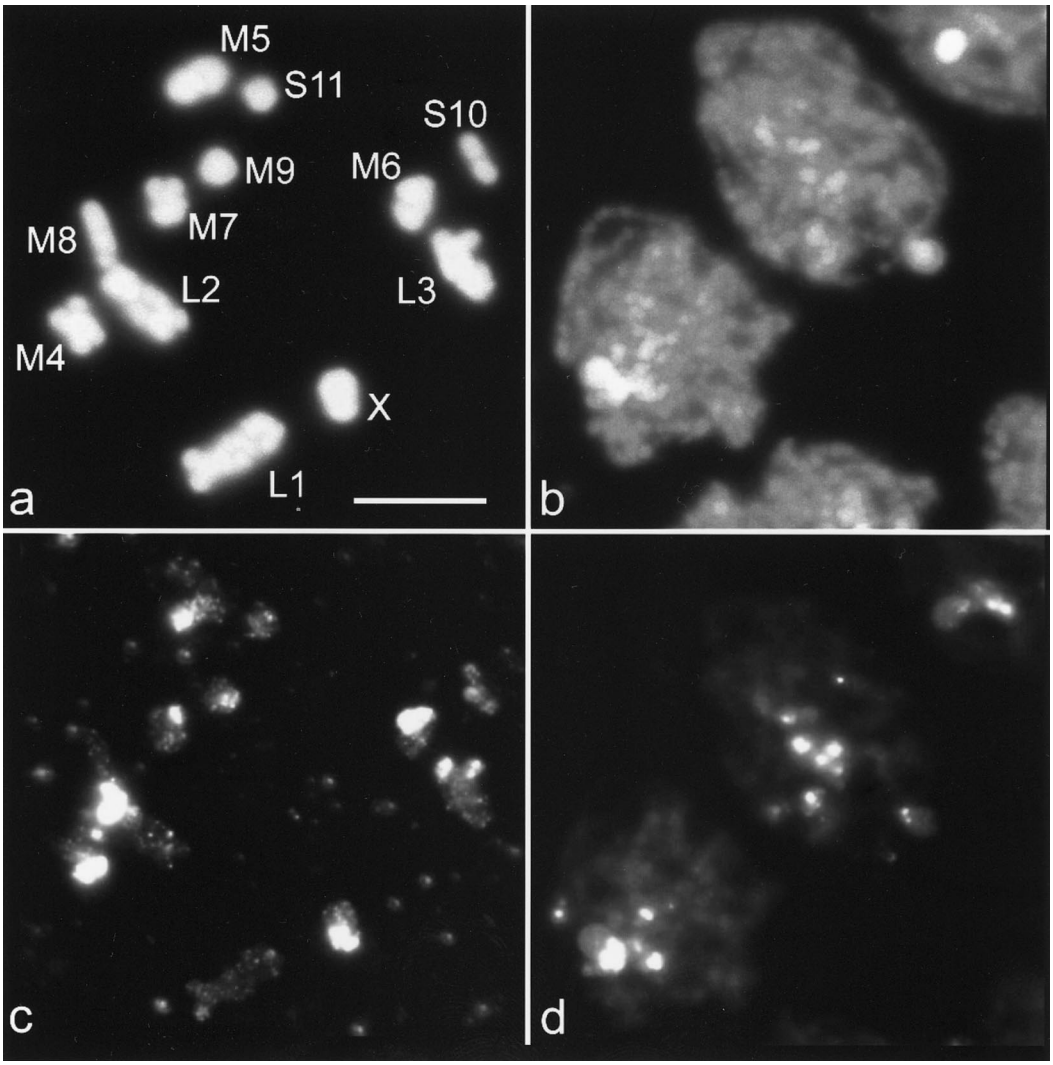
Fig. 2. Sites of in situ hybridisation of the cytochrome oxidase I (COI) mitochondrial sequence (yellow-green fluorescence; *d*, *e*, *f*) to orthopteran nuclei stained with DAPI (4′,6-diamidino-2-phenylindole, blue; *a*, *b*, *c*). (*a*, *d*) Metaphase I of *C. p. parallelus.* Centromeric sites are labeled as well as one telomeric site on bivalent L2 and both on M6. The X univalent is lower left; (*b*, *e*) Diplotene of *S. gregaria*. The X chromosome is out of the frame but all bivalents are present. Labeled sites are close to a single telomere of eight bivalents (and the X chromosome); (*c*, *f*) half anaphase I of *Italopodisma* sp. showing label dispersed over the chromosomes with some chromosomes showing less label. Bar = 10 μm.



in both subspecies and *C.p. parallelus* has an additional site distally on the X chromosome (Figs. 1*a*, *1d*). The same probe localizes to two and three discrete NORs in *S. gregaria* and *Italopodisma* sp., respectively, also indicating that the hybridisation conditions employed give an accurate representation of rDNA sites. The results seen in *S. gregaria* are consistent with silver-staining observations (Fox and Santos 1985). While *Italopodisma* sp.'s NORs have not previously been studied, those of a closely related Arctic–Alpine species, *Podisma pedestris* have. In one race of *P. pedestris* only two NORs have been described (Gosalvez et al. 1988), and within races rDNA polymorphism has been documented (Dallas et al. 1988). The proximal location of the X-NOR in *Italopodisma* sp. originating from Gran Sasso in Italy (Figs. 1*b*, 1*e*) is interesting in that Pyrenean *P. pedestris* populations show this X chromosome type, while Alpine populations of *P. pedestris* have a distal X-NOR (Gosalvez et al. 1988*b*). Further investigation of the chromosome differences across this species group could yield more information about the relationships of various species races and assist in the determination of Pleistocene range changes and colonisation of European mountain habitats by these species.

The COI sequence localizes to the nucleus of the orthopteran species *C. parallelus*, *S. gregaria* and *Italopodisma* sp. (Fig. 2). In two of these species, *C. parallelus* (Figs. 2*a*, 2*d*) and *S. gregaria* (Figs. 2*b*, *2e*), the strength of the signal indicates that the COI sequences are repeated multiple times in clusters. Probing with the single copy *hox* gene in *S. gregaria* yields no signal. This gives a striking comparison with the strength of signal observed with the

Fig. 3. In situ hybridisation of the mitochondrial control region (red fluorescence; *c*, *d*) to *S. gregaria* nuclei stained with DAPI (blue; *a*, *b*). (*a*, *c*) Diplotene showing distal labeling at one end of most bivalents and the X univalent. The pattern is similar to that seen with the COI probe; (*b*, *d*) Zygotene nuclei showing clustering of labeled sites. Bar = 10 µm.



COI probe. In *C. parallelus*, the repeats are localized around the centromeric regions, as well as at two minor sites distally on chromosomes L2 and M6. In *S. gregaria*, the sequences are repeated close to the telomeres of most, but not all, of the chromosomes in the complement. In *Italopodisma* sp., the sequence is apparently spread thoughout the genome (Figs. 2*c*, 2*f*).

The COI sequence, which has been generally accepted as simply mitochondrial in recent years, and used to construct phylogenies and deduce evolutionary and colonisation events, has nuclear copies in all three species studied. Not only are there nuclear copies, but there appear to be numerous repeats, at least in the genomes of *C. parallelus* and *S. gregaria*, where strong localized signal is observed. The sequence repetition could have arisen from insertion of mitochondrial DNA into the nucleus close to a repetitive element (e.g., Wakasugi et al. 1985). The cytoplasmically derived sequence would then be amplified along with its adjacent sequence. Candidate centromeric and telomeric repeats of *C. parallelus* and *S. gregaria*, respectively are known. The spread of sites between chromosomes may be a result of particular nuclear rearrangements, for example, if there is aggregation of particular chromosomal regions at some points in the cell cycle (Rabl 1885). The clustering of COI and control region sites in some zygotene and pachytene nuclei of *S. gregaria* shows that telomeres associate closely at some stages of development.

Several ideas have been put forward for the mode of transfer of organellar sequences, including mediation via retrotransposon sequences, integration of mitochondrial DNA into the nucleus particularly during early embryogenesis, when the number of mitochondria can be huge (Jacobs et al. 1983), and fusion of organellar membranes (Ellis 1982; Stern and Lonsdale 1982). In humans, nuclear integration of mitochondrial DNA close to putative retroviral sequences suggest a possible means of transfer of DNA (Wakasugi et al. 1985).

The COI sequence is localized to different parts of the karyotype in the different species (Fig. 2). This implies that there has not been a single ancestral transfer of the region

from mitochondrion to nucleus, followed by fixation. Given the plasticity and mobility of parts of eukaryotic genomes, it may be more likely that the sequence was transferred to the nucleus several times independently during evolution of the orthopterans. Independent transfers of mtDNA have previously been shown in aphids (Sunnocks and Hales 1996) and in birds (Sorensen and Fleischer 1996). Whether the transfer to nucleus from mitochondrion is monophyletic in origin or not, there has probably been rearrangement and amplification of the sequence since the transfer to the nucleus in the three genera studied. Interestingly, in situ hybridisation shows that the AT-rich control region of *S. gregaria* (Fig. 3) localizes in the same chromosomal domains as COI in the species examined. It is possible that both the control region and COI may have been translocated together into the nucleus as a single piece of DNA, carrying both sequences, more than 3 kb long. The transfer of large stretches of cytoplasmic sequence to the nucleus has been documented, including a region of 7.9 kb in the cat (Lopez et al. 1994).

Our results showing nuclear location of mitochondrial sequences are consistent with findings from sequence data, at least in the case of *S. gregaria* and *Italopodisma* sp. Regions of the COI gene derived from the mitochondrial fraction of *S. gregaria* DNA differ in sequence from those derived from the nuclear fractions (Zhang and Hewitt 1996). Sequence data from *P. pedestris*, a close relative of *Italopodisma* sp., yields anomalies consistent with more than one type of COI gene existing in total genomic DNA (D. Bensasson, University of East Anglia, personal communication). In the cat, Lopez et al. (1994) described the transfer of the 7.9-kb fragment of mitochondrial DNA to a specific site in the nucleus, and its amplification to between 38 and 76 copies.

There is evidence from sequence data that mitochondrial to nuclear transfer of the cytochrome oxidase II gene in legumes was mediated by an RNA intermediate (Nugent and Palmer 1991); the nuclear sequence is more similar to a putative RNA intermediate than to the original mitochondrial sequence. However, in this case, there was apparently nuclear integration of a single copy of the gene, most likely at one point in the evolution of the legumes, and this nuclear copy is transcriptionally active in at least some lineages. In contrast, numerous nuclear copies of the COI gene are present in *C. parallelus* and *S. gregaria*, and little is known about expression of the nuclear sequences or the mode of integration. Further sequence data comparing COI sequences from nuclear and mitochondrial fractions could yield information on the likelihood of RNA-mediated transfer and the potential for protein expression, although the difference in nuclear and mitochondrial genetic codes in insects is likely to preclude this.

It is not clear how much of the mitochondrial genome has been integrated into the nuclear genome in the orthopterans studied, nor is it clear how widespread is the phenomenon of interorganellar transfer of DNA in any animal or plant group. The integration of the cytochrome oxidase II gene in legumes suggests a single, ancestral transfer of DNA from mitochondrion to nucleus, with no amplification, while the data here indicate multiple transfers. Both our data and that of Lopez et al. (1994) show transfer of DNA to the nucleus, and its amplification. In general, there is no clear inference regarding the timing or frequency of interorganellar transfer of DNA (Stern and Palmer 1984; Lopez et al. 1994).

More information on other taxa would provide a better understanding of the situation. It would also be useful to allow the accurate interpretation of evolutionary processes and phylogenetic relationships. In situ hybridisation is a powerful tool in this type of study, allowing nuclear localization of probes from various origins. Sequence data from *C. parallelus*, *S. gregaria*, *Italopodisma* sp., and other grasshoppers could give information on the timing, frequency, and mode of intraorganellar transfer of DNA within orthopteran phylogeny.

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