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Phylogenetic relationships in West Mediterranean Scaritina (Coleoptera: Carabidae) inferred from mitochondrial COI sequences and karyotype analysis

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Abstract

The phylogenetic relationships of the West Mediterranean lineages of the subtribe Scaritina have been investigated by studying the mitochondrial cytochrome oxidase gene (COI) and the mitotic and meiotic chromosomes, including the localization of rDNA genes by fluorescence *in situ* hybridization. These sources of data are congruent and suggest the following conclusions: (1) the subgenus *Parallelomorphus* (genus *Scarites*) is a monophyletic group according to molecular and karyotypic data, in agreement with its geographical distribution and morphological characters; (2) in the same genus, the subgenus *Scallophorites* seems to be monophyletic but includes two well-separated lineages – one represented by *Scarites buparius* (Forster, 1771) and *Scarites occidentalis* (Bedel, 1895) (both with multiple sex chromosomes), and one represented by *Scarites hespericus* (Dejean, 1831); (3) the nominal subgenus *Scarites* s.s. represented by *Scarites eurytus* (Fischer, 1828), is cladistically more closely related to subgenus *Scallophorites* than to subgenus *Parallelomorphus*; (4) *Distichus planus* (Bonelli, 1813) belongs to a distinct clade that is well separated from *Scarites*, and shows more plesiomorphic states of COI sequence and chromosome number. In addition, the presence of two autapomorphies, such as rDNA sites located in the X chromosome, and an A + T rich intergenic spacer of about 120 bp between the stop codon of the COI gene and the tRNAleu, agree with the ranking of *Distichus* as a separate genus from *Scarites*.

Key words: Carabidae – COI – Coleoptera – *in situ* hybridization – karyotype – phylogeny – rDNA – Scaritina

Introduction

The subtribe Scaritina is a cosmopolitan group of fossorial beetles of Gondwanian origin (Jeannel 1941, 1946; Basilewsky 1973), that comprises several hundred species all over the world. Nine species are present in the West Mediterranean Basin, which according to the monographs of Bänninger (1937, 1938), correspond to the following lineages: *Distichus* (one species); *Scarites* subgenera *Scallophorites* (four species), *Scarites* s. str. (one species), and *Parallelomorphus* (syn. *Broscomorphus* Mots) (three species).

To date, no explicit phylogeny has been postulated for the subtribe Scaritina. Implicit relationships can be derived from the taxonomic criteria, based on the external morphology, defined by authors such as Bänninger (1937, 1938), Antoine (1955) and Nichols (1988), or the female genitalia (Ortuño 1996).

Karyotype analysis of Scaritina has also shown the existence of some traits of phylogenetic interest within the chomosomes. A multiple XXY sex chromosome system has been described in males of *Scarites occidentalis* (Serrano 1980) and *Scarites buparius* (Serrano 1984). *Distichus planus* has a 2n = 38 + X karyotype (Serrano et al. 1986) close to the proposed ancestral condition for many lineages of carabid beetles (2n = 36 + X; Serrano and Galián 1998). Three other species with varying numbers have also been karyotypically studied (Table 3).

The aim of this study was to obtain the first inference about phylogenetic relationships within the subtribe Scaritina, using molecular and cytogenetic data, and to compare the conclusions with the implicit hypotheses derived from current taxonomic criteria. We chose the West Mediterranean species as they include representatives of several lineages within the subtribe. For this purpose, a 489 bp fragment of the mitochondrial cytochrome oxidase gene (COI) was sequenced. This gene region has been used for reconstructing phylogenies of low-ranked taxa (e.g. Lunt et al. 1996), including some Coleopteran groups (Juan et al. 1995, 1996a,b). Karyotypic data were obtained for most species, including the chromosomal localization of rDNA genes by fluorescence *in situ* hybridization (FISH).

Materials and methods

Material

Individuals belonging to the seven species sampled in the study were collected in the localities listed in Table 1. Two of the three species belonging to the subgenus *Parallelomorphus* and three of the four belonging to *Scallophorites* were available for the study, as *Scarites subcylindricus* (Chaudoir 1843), and *Scarites striatus* (Dejean 1825), could not be collected.

DNA amplification and sequencing

Total genomic DNA was isolated by grinding the head and thorax of each specimen in liquid nitrogen and resuspending the homogenate in buffer (20 mM Tris, 10 mM EDTA, 0.5% SDS) containing proteinase K at a concentration of 50 μg/μl. The mixture was incubated overnight at 45 °C and the DNA was then purified with Promega (Madison, USA) 'Wizard clean up system' minicolumns following the manufacturer's instructions. The mtDNA template for sequencing was produced through polymerase chain reaction (PCR) with the primers 5′CCTACAGGAATTAAAATTTTTAGATGA3′ (situated in the second half of the COI gene, corresponding to the position 2410 of the mtDNA genome of *Drosophila yakuba*) and 5′TCCATTGCAC-TAATCTGCCATTTA3′ (located in the tRNAleu). These primers produce a 630 bp fragment at the 3′ end of the COI gene in all species analysed except *Distchus planus*, in which the fragment is 750 bp long. Each cycle of the PCR consisted of denaturation for 30 s at 94 °C, annealing for 1 min at 50 °C, and extension for 1 min at 72 °C. The cycle was repeated 35 times. Single stranded DNA was produced using Dynabeads M-280 (Dynal, Oslo, Norway) with one primer biotinilated. Sequences of a 489 bp fragment were obtained either manually or using a Pharmacia (Uppsala, Sweden) ALF automatic sequencer.

Phylogenetic analysis

Phenograms based on sequence distances were constructed using the neighbour-joining procedure (Saitou and Nei 1987) with the gamma distances for the Kimura two-parameter model (Kimura 1980), as implemented in the program package MEGA (Kumar et al. 1993).

Table 1. West Mediterranean species of Scaritina investigated in this study

Taxa	Localities
Distichus planus (Bonelli, 1813)	El Algar (Murcia), Dolores de Pacheco (Murcia)
Scarites (*Scallophorites*) *occidentalis* (Bedel, 1895) (*cyclops* Crotch, 1871)	Doñana (Cádiz)
S. (*Scallophorites*) *buparius* (Forster, 1771) (*pyracmon* Bonelli, 1813)	Doñana (Cádiz), Playa de Pego (Valencia), Salinas del Rasall (Murcia)
S. (*Scallophorites*) *hespericus* (Dejean, 1831) (*impressus* Fabricius, 1801)	Tarifa (Cádiz)
S. (*Scarites*) *eurytus* (Fischer, 1828)	Albatera (Alicante), Laguna del Hondo (Alicante)
S. (*Parallelomorphus*) *terricola* (Bonelli, 1813)	Salinas del Rasall (Murcia), Laguna del Hondo (Alicante)
S. (*Parallelomorphus*) *laevigatus* (Fabricius, 1792)	Playa de Calblanque (Murcia), Torrevieja (Alicante)

Parsimony analysis of the sequences was carried out using PAUP version 3.1.1 (Swofford 1993). Different character weighting of transversion versus transition and conserved first and second codon positions against third codon position were examined. Bootstrap tests (Felsenstein 1985) based on 500 replications were used to assess support of the various phyletic groups. Bootstrap frequencies were interpreted as heuristic levels of support rather than statistical significance levels.

The PHYLIP version 3.5 package was used to perform maximum likelihood analysis (Felsenstein 1993). Trees were rooted with the *Dyschiriodes subcylindricus* (Motschulsky, 1849) sequence, a species of the subtribe Dyschiriina which is closely related to Scaritina.

Cytogenetic analysis

Male gonads from adult beetles were used to obtain mitotic or meiotic chromosomes and nuclei as described in Galián et al. (1995). Reduction of background for *in situ* hybridization was carried out by placing small sections of the gonad in Eppendorf tubes with 20 µl of 60% acetic acid, pipetted up and down 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. *In situ* hybridization was performed as described previously (de la Rúa et al. 1996).

Results

Cytochrome oxidase I sequence data

A fragment of 630 bp was PCR-amplified by the chosen primers in all species except *Distichus planus* where a fragment 750 bp long was produced (not shown). Sequence analysis showed that this difference is due to an intergenic spacer of 120 bp placed between the stop codon of the COI gene and the tRNAleu sequence, which is outside the fragment used for the phylogenetic analysis. Figure 1 shows the aligned sequences of the 489 bp fragment of the mitochondrial COI gene obtained from seven West Mediterranean species of Scaritina, as well as one species of *Dyschiriodes* used as an outgroup. Insertions or deletions were not found in this region. In this gene segment, 127 positions were variable (including the outgroup species) of which 66 were phylogenetically informative. Gamma distances for the Kimura two parameter model were calculated using MEGA (Table 2). The greatest divergence was observed between *Scarites eurytus* and *Scarites laevigatus* (14.62%) and the lowest value (0.83%) between *S. laevigatus* and *Scarites terricola*.

Comparison of the inferred amino acid sequences in *Scarites* and *Distichus* using the mtDNA code for *Drosophila melanogaster* (de Bruijn 1983) shows that the polypeptide is highly conserved (not shown). Considering only the ingroup there are 23 of 163 sites that exhibit variation, and this number increases to 33 if *Dyschiriodes* is included. Some replacements define particular taxa. For instance, in amino acid position number 112 all species have isoleucine except the two species of the subgenus *Parallelomorphus*, *S. terricola* and *S. laevigatus*, that have valine; or in position 122 all *Scarites* and *Distichus* species have phenylalanine whereas *Dyschiroides* sp. has leucine.

An exhaustive search using PAUP assuming equal weight for all characters produced one most parsimonious tree of 194 steps (consistency index = 0.804, retention index = 0.624). The bootstrap tree after 500 replications is shown in Figure 2. The same topology is obtained when weighting three- or five-fold transversions/transitions. The use of only first and second codon positions, which is approximately equivalent to using non-synonymous substitutions, results in a loss of phylogenetic information, giving a node with three branches *buparius* + *occidentalis* + *hespericus* plus *euritus* plus *laevigatus* + *terricola*.

The neighbour-joining option of MEGA was used to construct a phenogram for complete distance values calculated using the gamma distance for the Kimura two parameter model (not shown). The topology of this tree is the same as that of the single most parsimonious tree, which in turn proved to be identical to the maximum likelihood topology calculated in PHYLIP (using empirical base frequencies and a transition/transversion ratio of 1.5, ln likelihood = −1601.51, 70 trees examined). In both trees the weakest bootstrap value is in the node that separates *eurytus* from the clade formed by the species of the subgenus *Scallophorites*.

Karyotype analysis

The chromosome number and sex chromosome mechanisms of all species are listed in Table 3 and also shown in Figure 2. *Scarites buparius* and *Scarites occidentalis* (subgenus *Scallophorites*) show some similarities, such as three sex chromosomes of large size (Figs 3a, b). In *Scarites hespericus and S. eurytus*, the chromosome number is higher and there is only one X chromosome (Figs 3c, d). Karyograms of species belonging to the subgenus *Parallelomorphus* show the highest numbers, with an odd and large chromosome that has been identified as the single X heterosome (Figs 3e, f). In *S. laevigatus* it is clearly the largest member of the karyotype. *Distichus planus* also has a large X heterosome. Some autosomal pairs of large size (third and fourth) have asymmetrical arms (Fig. 3g).

Meiosis follows an orthodox pattern (Fig. 4). The few species examined show a number of interstitial chiasmata higher than

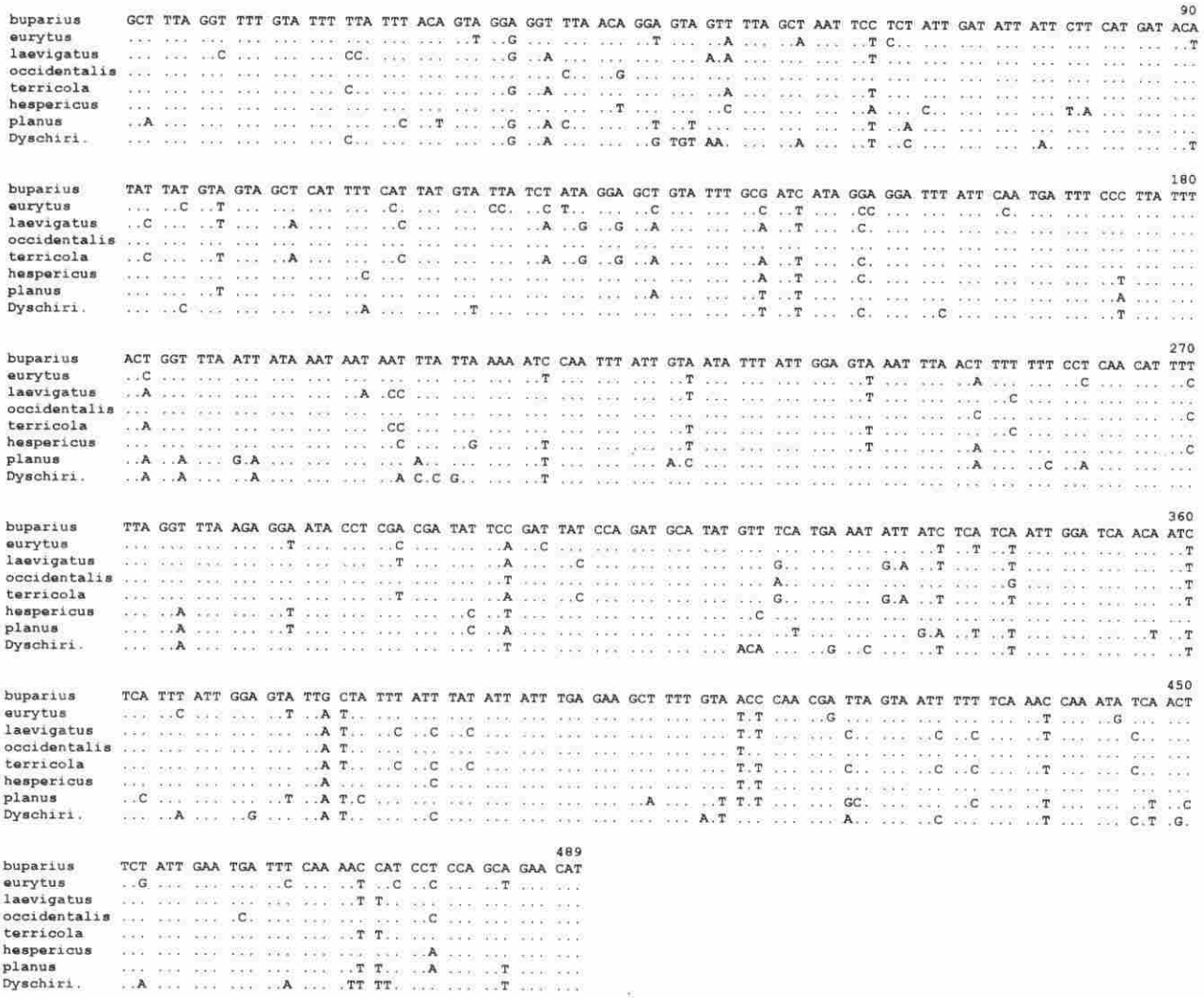

Fig. 1. Sequence of a 489 bp region of mitochondrial cytochrome oxidase I from seven West Mediterranean species of Scaritina and *Dyschiriodes subcylindricus*. Dots indicate identity to sequence of *Scarites buparius*

Table 2. Matrix of interspecific divergence (Gamma distance for the Kimura two-parameter model) in the mitochondrial COI fragment among seven West Mediterranean species of Scaritina and *Dyschiriodes subcylindricus* (Dyschiriini)

	S. occi.	*S. hesp.*	*S. eury.*	*S. laev.*	*S. terr.*	*D. plan.*	*Dyschir.*
S. buparius	0.0278	0.0623	0.1221	0.1177	0.1064	0.1352	0.1475
S. occidentalis		0.0672	0.1162	0.1239	0.1124	0.1323	0.1594
S. hespericus			0.1250	0.1314	0.1198	0.1440	0.1680
S. eurytus				0.1462	0.1342	0.1470	0.1838
S. laevigatus					0.0083	0.1620	0.1678
S. terricola						0.1499	0.1617
D. planus							0.1677

the average in most carabid groups, in which only (or mostly) distal chiasmata are seen. In the species that showed the best chromosome spreads (e.g. *S. occidentalis*, Fig. 4b), heteromorphic bivalents and B-chromosomes can be found. Variations in chromosome number have been also found in *S. buparius* (Serrano 1980; unpublished results). These polymorphisms are indicative of a karyotypic dynamism which agrees with the numerical and morphological differences that exist between species. Two species of *Scallophorites*, *S. buparius and S. occidentalis*, show a sex trivalent which co-orientates regularly whereas *S. hespericus* show only a single X chromosome. The single sex univalent in the other species can frequently be seen lying outside the metaphase plate (Figs 4d, f), as commonly found in other carabid groups with XO males.

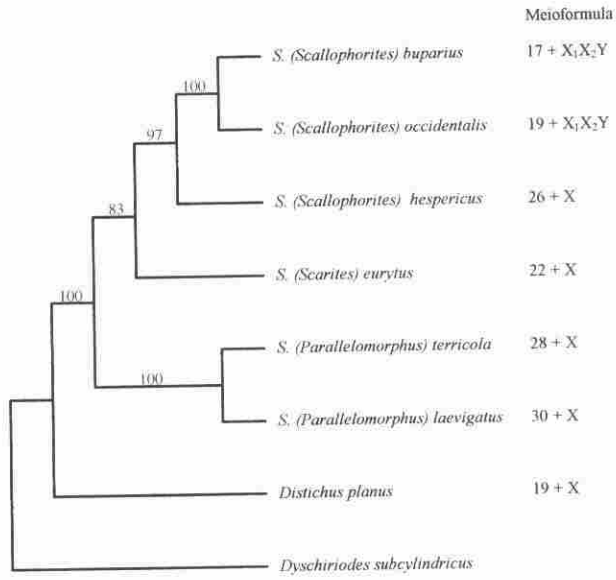

Fig. 2. Single most parsimonious tree for seven West Mediterranean species of Scaritina plus the outgroup. Numbers on branches indicate the percentage of bootstrap for 500 replicates using a exhaustive search. The male haploid chromosome number (meioformula) of each species is indicated to the right

(n = 19 + X) and with two (n = 19) fluorescent yellow signals (Fig. 5e).

Discussion

Phylogeny reconstruction from the COI sequence data using different inference methodologies and weighting strategies shows a robust topology for the relationships of West Mediterranean Scaritina. The very low substitution rate estimated between the sequences of the species of the subgenus *Parallelomorphus*, *S. terricola* and *S. laevigatus* (0.8%), indicates a close relationship of these species. Their high chromosome number (also found in Indian species of *Parallelomorphus*; Table 3) and the characters of the female genitalia reported by Ortuño (1996), suggest that this subgenus is a well-defined monophyletic group.

Two species of the subgenus *Scallophorites*, *S. buparius* and *S. occidentalis*, are closely related in all the characters considered. They constitute a monophyletic group with a high bootstrap value for this node (100) and low substitution rate (0.028) of the COI sequence. Both species share an apomorphy in the male sex-chromosome system (XXY) which is rare among carabid beetles, and show almost identical female genitalia (Ortuño 1996). *Scarites hespericus* is cladistically related to the former species (Fig. 2). However, the lack of the distinctive XXY sex trivalent found in *S. buparius and S. occidentalis*, its higher chromosome number, the female genitalia (Ortuño 1996), and its ecological preferences (it inhabits red compact clayish soils instead of sandy soils) clearly set *S. hespericus* apart from the closely related pair of *S. buparius and S. occidentalis*. Karyotypic results, the presence of more rDNA clusters, the external morphology and the female genitalia indicate that *S. eurytus* belongs to a different lineage from that formed by the precedent species, a conclusion that is in agreement with the position of this species in both the parsimony (Fig. 2) and distance trees. Data on the COI sequence indicate that the lineage of *S. eurytus* is more akin to *Scallophorites* than to *Parallelomorphus*.

Distichus planus is the sister-group of the other West Mediterranean Scaritina analysed here. Similar evolutionary distances separate this species from both the other species of the subtribe and the outgroup represented by *Dyschiriodes* (Table 2), and support its placement in a different genus as currently proposed by authors after Bänninger (1937, 1938). The presence of rDNA sites in the X chromosome and the intergenic spacer between the COI and tRNAleu are also pec-

Table 3. Karyotypic data of Scaritina

Taxa	Diploid no.	Haploid no.	References
Distichus planus (Bonelli, 1813)	38 + X	19 + X	1, 2, this paper
Scarites (*Scallophorites*) *occidentalis* Bedel, 1895	41	19 + X_1X_2Y	3, this paper
S. (*Scallophorites*) *buparius* (Forster, 1771)	37 – 39 + Bs	17 + X_1X_2Y	4, this paper
S. (*Scallophorites*) *hespericus* Dejean, 1831	53	26 + X	this paper
S. (*Scarites*) *eurytus* Fischer, 1828	45	22 + X	this paper
S. (*Scarites*) *subterraneus* Fabricius, 1775	–	18 + X	5
S. (*Parallelomorphus*) *terricola* Bonelli, 1813	57	28 + X	this paper
S. (*Parallelomorphus*) *laevigatus* Fabricius, 1792	61	30 + X	this paper
S. (*Parallelomorphus*) *inconspicuus* Chaudoir, 1855	52	25 + XY	6
S. (*Parallelomorphus*) *indus* Olivier, 1795	52	25 + XY	6

References: 1, Serrano (1981); 2, Serrano et al. 1986; 3, Serrano 1984; 4, Serrano 1980; 5, Smith (1960); 6, Yadav et al. (1987).

Localization of rDNA clusters in *Scarites* and *Distichus*

Fluorescence *in situ* hybridization shows four sites of rDNA hybridization, autosomally located in *S. terricola*, *S. laevigatus*, *S. buparius*, *S. occidentalis* and *S. hespericus*. In *S. terricola*, the yellow hybridization signal is located in the largest pair and in a medium-sized pair, occupying the entire short arm (Fig. 5a). In all these species spermatid nuclei show two signals. *Scarites eurytus* shows a different pattern, having rDNA clusters located in three autosomal pairs, ranging from large to medium size and forming only one chiasma. The yellow hybridization signal is located in the non-pairing arm (Fig. 5c). Spermatid nuclei of this species show three hybridization sites (Fig. 5f). *Distichus planus* also differs from the pattern elucidated above, in having rDNA clusters in the single X heterosome as well as in two autosomal pairs. In first metaphase plates the single X is situated peripherally to the autosomes and is clearly identified, showing a strong yellow signal in all metaphases analysed (Fig. 5d). Spermatid nuclei are of two types, with three

Fig. 3. Male karyogram of: (a) *Scarites buparius*, 2n = 34 + X_1X_2Y; (b) *S. occidentalis*, 2n = 38 + X_1X_2Y; (c) *S. hespericus*, 2n = 52 + X; (d) *S. curytus*, 2n = 44 + X. Sex chromosomes are figured to the right. Bar equals 5 μm. Male karyogram of: (e) *Scarites terricola*, 2n = 56 + X; (f) *S. laevigatus*, 2n = 60 + X; (g) *Distichus planus*, 2n = 19 + X. Sex chromosomes are figured to the right. Bar equals 5 μm

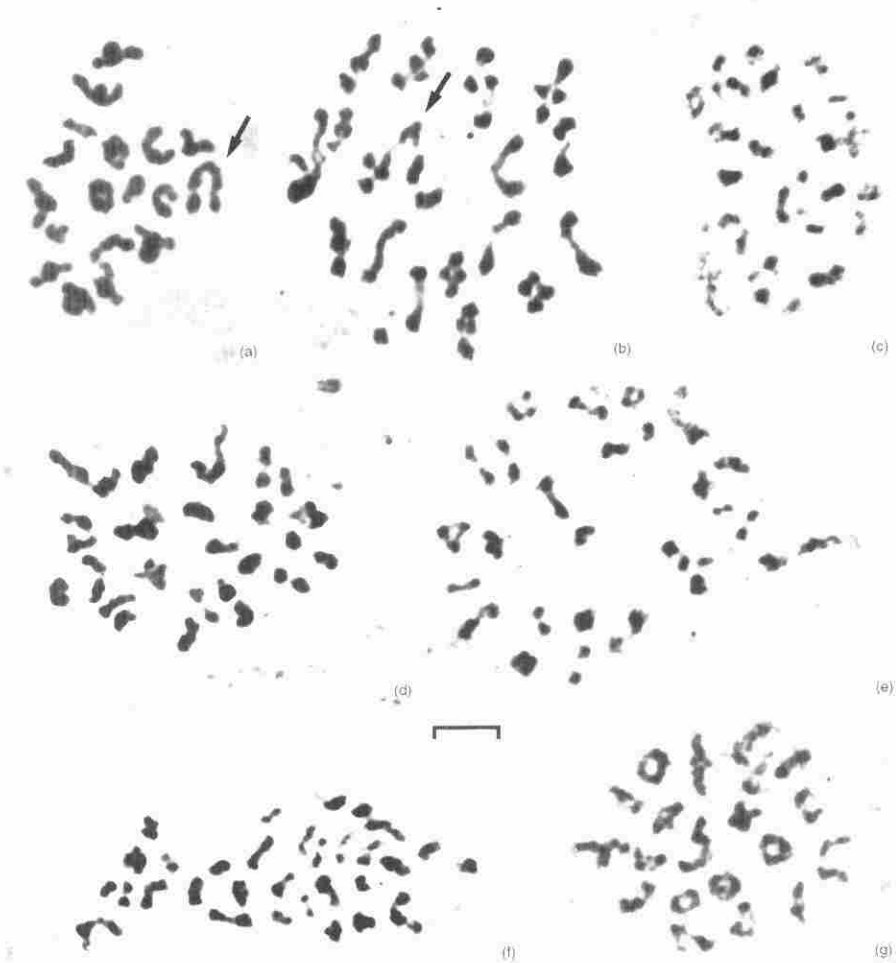

Fig. 4. Meiotic chromosomes of West Mediterranean Scaritina after conventional orcein or Giemsa staining: (a) *Scarites buparius*, $n = 17 + X_1X_2Y$; (b) *S. occidentalis*, $n = 19 + X_1X_2Y$; (c) *S. hespericus*, $n = 26 + X$; (d) *S. eurytus*, $n = 22 + X$; (e) *S. terricola*, $n = 28 + X$; (f) *S. laevigatus*, $n = 30 + X$; (g) *Distichus planus*, $n = 19 + X$. Bar equals 5 μm

uliarities in this species that deserve further investigation in other species of the genus. Other intergenic spacers (between tRNAleu and COII gene) have been described previously in *Apis mellifera*, where they are supposed to include one origin of replication (Cornuet et al. 1991), and in the Dipteran *Rhagoletis* (Smith and Bush 1997). The characterization and significance of this spacer will be presented elsewhere (Galián et al. in preparation).

The taxonomic implications of the above conclusions are diverse. First, *Distichus* may well be considered as a distinct genus from *Scarites*. Second, *Parallelomorphus* is a well-defined subgenus within the large genus *Scarites*. Third, the subgenus *Scallophorites* seems to be monophyletic. However, additional species should be examined in order to further elucidate the relationship between the group here represented by *S. buparius and S. occidentalis* (and probably including further species from the Mediterranean basin) and that represented by *S. hespericus* (and probably including further species from Africa and Middle East). Fourth, the subgenus *Scarites* s.s. seems to be more closely related to *Scallophorites* than to *Parallelomorphus*, but additional species need to be studied.

The nucleotide sequence of the eight taxa determined here have been deposited with the GenBank Data Library under Accession Nos. AF042674-AF042681

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Zusammenfassung

Phylogenetische Verwandtschaftsbeziehungen im westmediterranen Subtribus Scaritina (Coleoptera: Carabidae) auf Grund von Vergleichen von mitochondrialen COI-Sequenzen und der chromosomalen Karyotypen

Die phylogenetische Verwandtschaftsbeziehungen zwischen westmediterranen Arten des Subtribus Scaritina wurden durch die Untersuchung des mitochondrialen Cytochromoxidase-Gens (COI) und der mitotischen und meiotischen Chromosomen – hier besonders durch die Lokalisierung der rDNA Genen mit Hilfe der Fluoroszenz-*in situ* - Hybridisierung, analysiert. Die aus diesen Datenquellen abgeleiteten Ergebnissen sind kongruent und führen zu folgenden Schlußfolgerungen: 1. Die Untergattung *Parallelomorphus* (Gattung *Scarites*) muß auf Grund der molekularen und karyologischen Befunde als monophyletische Gruppe angesehen werden, was mit ihrer geographischen Verbreitung und den morphologischen Merkmalen gut übereinstimmt. 2. Die Untergattung *Scallophorites* derselben Gattung scheint zwar monophy-

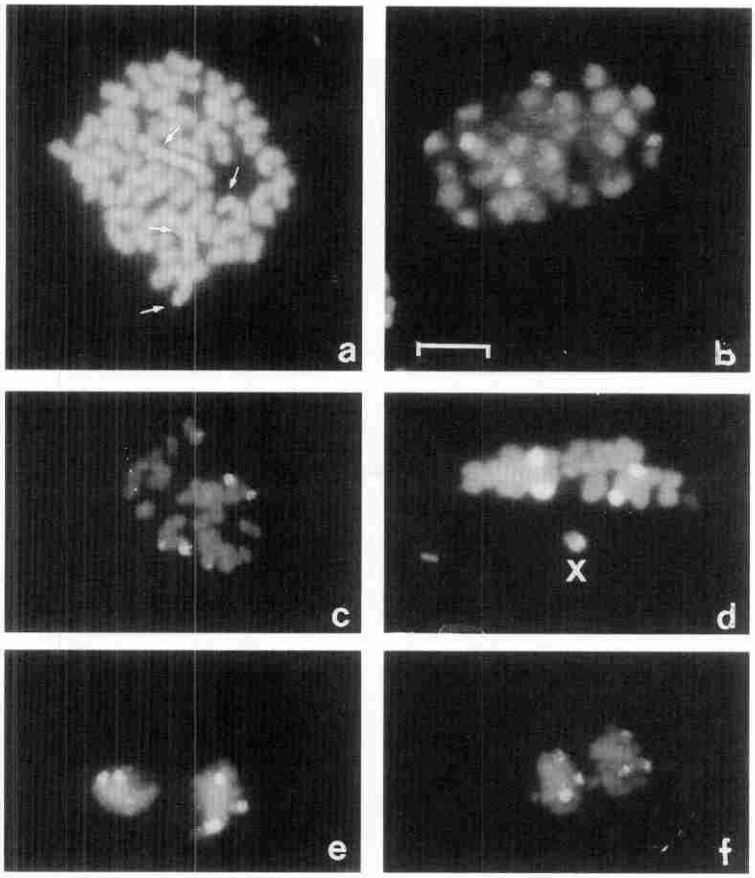

Fig. 5. Fluorescence *in situ* hybridization of chromosomes and nuclei of West Mediterranean Scaritini with a PCR-amplified ribosomal probe obtained from *S. occidentalis*: (a) *Scarites terricola*, spermatogonial mitosis showing two pairs of chromosomes with rDNA sites; (b) *S. occidentalis*, nongonial cell showing four sites of hybridization. (c) *S. eurytus*, metaphase I plate with three bivalents showing rDNA clusters. (d) *Distichus planus*, metaphase I plate showing two autosomal pairs plus the X heterosome (peripherally located) carrying rDNA genes; (e) *D. planus*, sister spermatic nuclei with two (without X) or three (with X) hybridization sites; (f) *S. eurytus*, spermatid nuclei showing three sites of hybridization each. Bar = 5 μm

letisch zu sein, umfaßt aber zwei deutlich getrennte Gruppen: In einer Gruppe finden sich *Scarites buparius* (Forster, 1771) und *Scarites occidentalis* (Bedel, 1895) (beide mit multiplen Geschlechtschromosomen), in der anderen *Scarites hespericus* (Dejean, 1831). 3. Die nominelle Untergattung *Scarites* s.s., durch *Scarites eurytus* (Fischer, 1828), vertreten, ist kladistich näher mit der Untergattung *Scallophorites* als mit der Untergattung *Parallelomorphus* verwandt. 4. *Distichus planus* (Bonelli, 1813) bildet eine eigene Gruppe, die von *Scarites* deutlich getrennt ist und plesiomorphe COI-Sequenzen und Chromosomenzahlen aufweist. Außerdem rechtfertigt das Vorhandensein von zwei Autapomorphien (rDNA-Sites am X-Chromosom und ein A + T-reicher intergenischer Spacer von $\approx$ 120 bp zwischen dem Stopcodon des COI-Gens und dem tRNAleu-Locus) die Zuordnung von *Distichus* zu einer eigenen Gattung.

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