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The use of mitochondrial DNA sequences in insect taxonomy: examples from the *Scathophagidae* (Diptera, Calyptratae)

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INTRODUCTION

The use of mitochondrial DNA as molecular markers for a wide range of taxonomic, phylogenetic, population and evolutionary investigations in animals has become well established (AVISE *et al.* 1987, HILLIS 1987, MORITZ *et al.* 1987, PACKER 1989, QUICKE 1993, AVISE 1994, SIMON *et al.* 1994, HILLIS *et al.* 1996). The positive attributes of mitochondrial DNA can be summarised as follows: (i) Its small size is associated with relative manageability and ease of extraction. (ii) Most cells contain many mitochondria, so that a significant quantity of mtDNA is available even from small tissue samples. (iii) A general conservation of gene order and composition across metazoa. (iv) A range of mutational rates in different regions of the molecule. (v) Because of its maternal inheritance, differences that occur in mtDNA are due entirely to mutation and are not the result of independent assortment or recombination (QUICKE 1993, MORITZ *et al.* 1987, WOLSTENHOLME 1992, AVISE *et al.* 1987).

The mtDNA in higher animals is a circular, double-stranded molecule. It comprises approximately 16'000 base-pairs encoding two rRNAs, 22 tRNAs and usually 13 other genes, mostly coding for proteins involved in the electron transport system located on the inner mitochondrial membrane. Animal mitochondrial genes lack introns and intergenic sequences are generally small or lacking. Also absent are repetitive DNA and pseudogenes. Further, the genetic code employed in mitochondria is slightly different from that of nuclear genes. (CLARY & WOLSTENHOLME 1985, AVISE *et al.* 1987, MORITZ *et al.* 1987, WOLSTENHOLME 1992, CROZIER & CROZIER 1993).

Although mtDNA-sequence data have proven valuable in phylogenetic analysis, gene choice is of crucial importance (SIMON *et al.* 1994, LUNT *et al.* 1996). Among the coding genes in the mitochondria genome, the subunit I of the cytochrome oxidase (COI) gene possesses features making it particularly suitable for evolutionary studies (LUNT *et al.* 1996). First, it has been well-studied at the biochemical level and its size and structure appears to be conserved across all animals investigated. Then, because the COI gene is one of the largest protein-coding genes in the animal mitochondrial genome, it is possible to amplify and sequence a large portion of DNA within the same functional complex. Furthermore, highly conserved and variable regions are closely associated.

Using examples taken from the *Scathophagidae* (Diptera), we illustrate the usefulness of mtDNA sequences in elucidating taxonomic and phylogenetic questions. Scathophagids flies, with more than 250 species, are mainly confined to the Holarctic region. Only about 5 species occur south of the equator, in South America and Africa, mostly at high altitudes, whereas in the Oriental region one species occurs in the Malay Peninsula (VOCKEROTH 1956, VOCKEROTH 1989). Individuals of most species feed on vertebrate dung or decomposing carcasses, performing the ecologically-important function of resource recycling. One species, *Scathophaga stercoraria*, has been used extensively to investigate questions in animal ecology and evolution (SIGURJONSDOTTIR & PARKER 1980, AMANO 1983, PARKER & SIMMONS 1991, SIMMONS & WARD 1991, WARD & SIMMONS 1991, SIMMONS & PARKER 1992, PARKER *et al.* 1993, WARD 1993, WARD & HAUSCHTECK-JUNGEN 1993; MÜHLHÄUSER *et al.* 1996, SIMMONS *et al.* 1996, BLANCKENHORN 1997, OTRONEN *et al.* 1997, BLANCKENHORN 1998, WARD 1998). However, the taxonomy and phylogeny of this family still remain unclear. The use of morphological characters, including the structure of male genitalia which seems to be very variable for some species are usually enough for the correct identification of genera and species but are still insufficient to infer precise phylogeny. The integration of the new molecular data with previous morphological studies should provide therefore a powerful tool for the inference of correct phylogeny of this fly family.

MATERIALS AND METHODS

After DNA extraction and standard PCRs, a portion of 810 bp of the terminal region of the COI gene was sequenced in 40 Scathophagid species covering a wide geographic area, as well as a diverse spectrum of ecological habitats. Moreover, three other flies species of the *Mus-*

coidea superfamily, *Musca domestica* (family *Muscidae*), *Lasiomma seminitidum (Anthomyiidae)* and *Fannia armata* (*Fanniidae*) were also sequenced. Outgroup comparisons were made with published COI gene sequence of *Drosophila yakuba* (EMBL database access X03240), and translations to amino acid sequences used the *Drosophila* code (DE BRUIJN 1983). Sequence data were analysed by pairwise sequence alignment and by multi alignment with the Lasergene program Megalign (DNAstar 1994) and phylogenetic analysis were performed using MEGA (Molecular Evolutionary Genetics Analysis 1.01, KUMAR *et al.* 1993). Several Neighbour Joining trees were generated using different methods for distance estimation. Some Maximum Parsimony trees obtained with heuristic search were also examined.

RESULTS AND DISCUSSION

The topologies of the several phylogenetic trees generated are overall very similar (Fig. 1). In general, molecular data are in agreement with previous morphological information. But some peculiarities reveal discrepancies between the two approaches. Within the *Scathophagidae* two subfamilies are currently recognised: the *Delinae* (represented here by the species *Delina nigrita* and *Phrosia albilabris*) and the *Scathophaginae* (GORODKOV 1986, VOCKEROTH 1989). However, various authors have proposed a different number of subfamilies or phylogenetic groups (ranging from 2 to 5; SACK 1976). The molecular data do not confirm the separation in 2 subfamily-groups currently recognised and the relationships among the groups proposed in the past

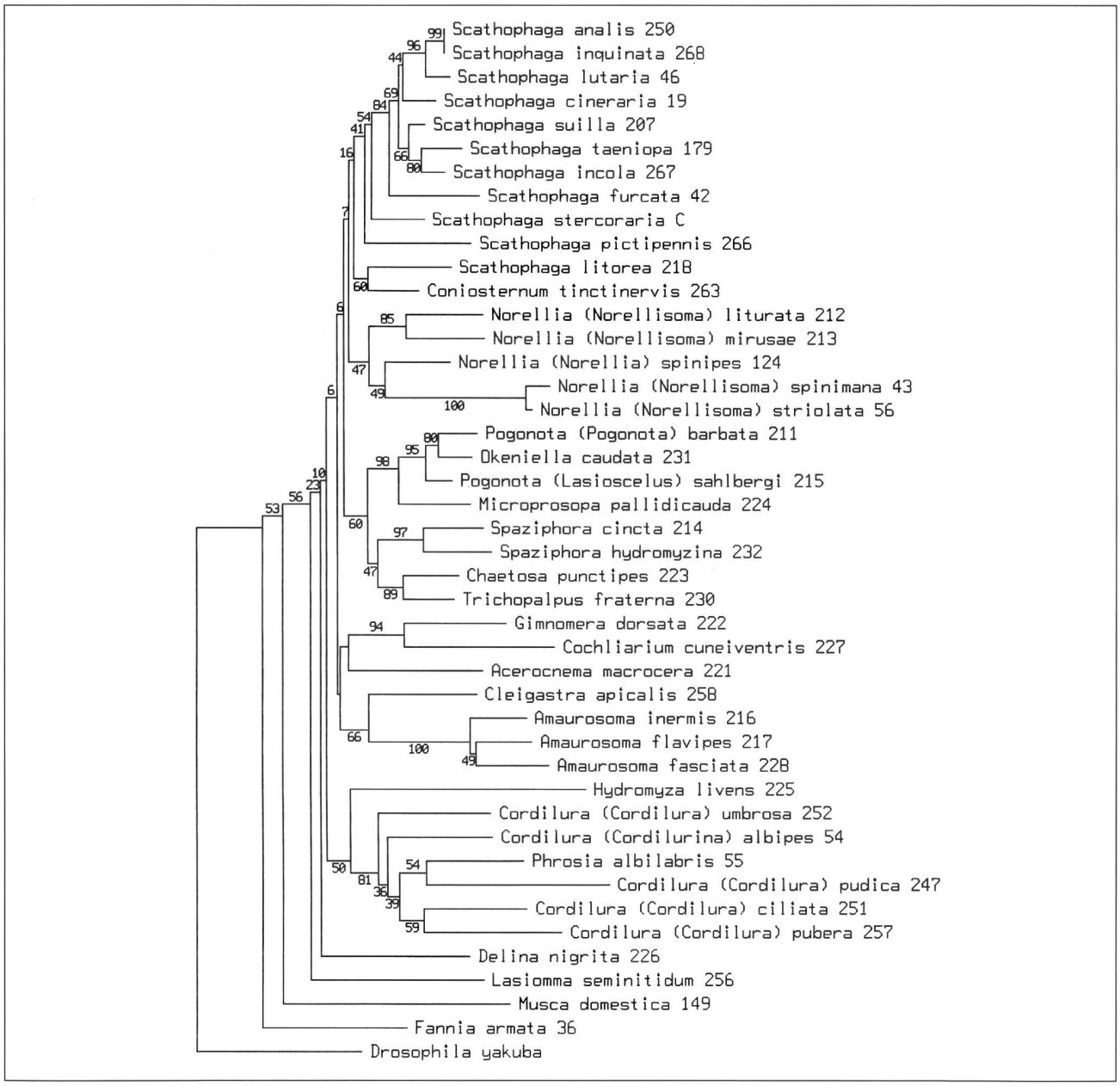

Fig. 1 - Rooted Neighbour Joining tree for 40 Scathophagids species and 3 other Muscoidea species (*Musca domestica* belongs to the *Muscidae*, *Lasiomma seminitidum* to the *Anthomyiidae*, and *Fannia armata* to the *Fanniidae*) based on the Tamura-Nei model and gamma corrected distances (with default gamma parameter a=0.5). *Drosophila yakuba* was chosen as outgroup. Bootstrap values for 500 replicates are shown.

are not always confirmed. Only the *Delina* genus appears sometimes to be isolated relative to the other genera. Nevertheless, this information is not supported by all the trees generated and the bootstrap values are usually low. Concerning the genus *Phrosia*, its membership of the *Delinae* is controversial even from the morphological point of view. As a matter of fact some authors consider this genera as a *Scathophaginae* and cannot understand why *Phrosia* should be placed in the *Delinae* subfamily (F. Püchel, personal communication 1997). In particular, adult individuals of *Phrosia albilabris* are morphologically very similar to the species belonging to the genus *Cordilura* (R. Vockeroth, personal communication 1998). Molecular data not only support the idea that *Phrosia* should be considered as a true *Scathophaginae* but also place this species unequivocally within the *Cordilura* genus.

Pogonota, *Lasioscelus* and *Okeniella* were once treated at the genus rank and considered as belonging to the same phylogenetic group (HACKMAN 1956). Modern taxonomists have now placed *Lasioscelus* as a subgenera of *Pogonota* (GORODKOV 1986) The molecular data confirm the relationship among the 3 groups relative to the *Scathophagidae*. However, it seems not correct to consider *Lasioscelus* as a subgenera of *Pogonota*, since no tree supports this interpretation. Even the generally high bootstrap values indicate a more strict relationship between *Pogonota* and *Okeniella* than between *Pogonota* and *Lasioscelus*.

Scathophaga taeniopa and *S. suilla*, are morphologically very similar species. In particular, a detailed study based on traditional morphological characters and comparing also the genitalia of the two species concluded that *S. taeniopa* and *S. suilla* are identical (SIFNER 1975, 1995). The major problem related to these two species is the risk of error by morphological determination, perhaps because of the lack of clearly distinctive chaetotaxy which could be applied to all the individuals. In this context the molecular data are very useful, allowing a sure characterisation of the specimens. Even if the case is not to be considered closed, the molecular data unequivocally suggest that *S. taeniopa* and *S. suilla* are different and must therefore be considered as distinct taxa.

S. analis, even if present in classical works on *Scathophagidae*, is considered as a doubtful species in the Catalogue of Palaearctic Diptera (GORODKOV 1986). Molecular data seems to indicate that it is probably a synonym of *S. inquinata*. In the determination key, the discriminating point is represented by the anterior cross-vein which should be dark-shadowed in *S. analis* but not in *S. inquinata* (SACK 1976). It is possible that the cross-vein character used to separate the two fly types is not valid, even if analysing the specimens from a morphological point of view, some other showy differences appear, like for example the colour of the shoulders. Perhaps under the name *S. analis* and *S. inquinata* entomologists have separated individuals belonging to the same species. The only way to clarify the question is to get sequence information generated from specimens which have been previously identified without any doubt by morphological characters.

In conclusion, as shown by these examples, the integration of the molecular data derived from mtDNA sequences, together with previous morphological studies, provides a keystone for the inference of correct phylogeny and taxonomy of an insect family.

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