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A molecular characterization of species and populations of *Dugesia gonocephala* s.l.

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ABSTRACT. We have employed a molecular approach to identify genomic characteristics in planarians belonging to *Dugesia gonocephala* s.l. In this paper we report the further characterisation of a repetitive DNA family (De1) that represents a useful molecular marker for establishing the genomic relationships among the species. We also describe the use of the molecular approach to obtain information about the genome organization of an asexual population of *D. gonocephala* s.l. from Montecristo Island, a natural park of the Tuscan Archipelago in the Tyrrhenian Sea. Specific DNA patterns have been individuated by using Southern blot analysis and the RAPD-PCR technique. These results, compared with those obtained in other species of *Dugesia gonocephala* s.l., indicate that some genomic differences characterise this geographically isolated planarian population. The possibility of a microspeciation event in the insular area is hypothesised.

KEY WORDS: Platyhelminthes, Tricladida, *Dugesia*, repetitive DNA, RAPD, phylogenetic relationships.

INTRODUCTION

Dugesia gonocephala s.l. includes a group of widely distributed planarian species, very similar in external features. Due to the important diagnostic value of the copulatory apparatus in the taxonomy of these organisms, the specific identification of the numerous asexual populations ascribable to *Dugesia gonocephala* s.l., is problematic. Karyotype information is usually of little help, because these planarians have chromosomes very similar in shape; aneuploidy and B-chromosomes can also be present.

Studies based on molecular analyses have recently allowed assigning asexual populations to a precise species by direct comparisons of genetic differentiation. Ribosomal DNA analysis results were particularly informative. In fact, both the chromosomal localization and the restriction pattern of 18S+28S rDNA (BATISTONI et al., 1999), as well as the sequence comparison of the internal transcribed spacer 1 (ITS1) region (BAGUÑÀ et al., 1999; BAGUÑÀ et al., 2001), revealed the presence of well-defined species-specific differences.

In addition, the characterisation of a highly repeated DNA family, conserved only in some species of the group (De1 family, originally isolated in *Dugesia etrusca* as a 1.4 kb *Hind*III fragment: BATISTONI et al., 1998), produced some information about their genomic relationships.

In this paper we report the cloning of a long fragment of a De1 unit. We also describe karyological and molecular characteristics of an asexual population of *D. gonocephala* s.l. from Montecristo Island.

MATERIAL AND METHODS

Animals and DNA extraction

Different species and populations belonging to *D. gonocephala* s.l. were used in this study. The precise locations where the animals were collected are given in BATISTONI et al. (1998).

Some specimens from Montecristo Island (Tuscan Archipelago, Tyrrhenian Sea, Italy) were kindly provided by Dr. M.A.L. Zuffi.

Genomic DNA was extracted from whole planarians as indicated by BATISTONI et al. (1998).

Karyological analysis

Chromosomes were prepared and analysed as described by DERI et al. (1999).

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Cloning of *Xba*I DNA fragments

*Xba*I-digested *D. etrusca* genomic DNA was electrophoresed on a 0.7% agarose gel. DNA fragments, at about 8kb, were recovered from the agarose with Qiaquick Gel Extraction kit (Qiagen) and cloned into the *Xba*I site of pGEM7Z(-) (Promega). On the basis of cross-hybridization with a previously cloned 1.4 kb *Hind*III repeat (p20 clone, BATISTONI et al., 1998), one of the obtained clones (p*Xba*I) was selected for the experiments and partially sequenced with the T7 DNA polymerase kit (Pharmacia).

Southern blot hybridizations

D. etrusca digested DNA was electrophoresed on a 0.7% agarose gel and blotted onto Hybond-N filters for hybridization experiments. Digoxigenin-labelled p20 and p*Xba*I inserts were used as probes. Hybridization conditions are described by BATISTONI et al. (1998).

RAPD analysis

RAPD procedure was essentially carried out according to WILLIAMS et al. (1990). Each PCR included about 25 ng of genomic DNA in a final volume of 20 µl, with 0.2 µM 10-mer oligonucleotide primer and 0.4 units of Taq DNA polymerase (Promega). An initial denaturation cycle (94°C, 4 min) was followed by 35 cycles (94°C, 1 min; 40°C, 1 min; 72°C, 2 min) and, finally, by an extension cycle (72°C, 4 min). Amplification products were electrophoresed on 1.5 % agarose gel; 1 kb DNA ladder (Gibco-BRL) was used as a molecular marker.

RESULTS AND DISCUSSION

Structure of De1 DNA elements

Our previous studies based on the presence/absence of a highly repeated DNA family (De1) dispersed throughout the genome of some *D. gonocephala* s.l. species, suggest that these sequences represent molecular markers that are important for making phylogenetic inferences (BATISTONI et al., 1998; 1999; BAGUÑÀ et al., 2000).

A Southern blot analysis of *D. etrusca*-digested DNA, using the previously cloned 1.4 kb *Hind*III De1 fragment as a probe (BATISTONI et al., 1998), indicated that this family is made up of long elements. *Kpn*I, *Pst*I, *Sal*I, *Sma*I and *Xba*I restriction enzymes produced single autoradiographic bands, between 8 and 12 kb (Fig. 1). The length of the De1 units should correspond to at least the longest band observed, i.e. about 12 kb.

In order to gain further insights into the structural characteristics and genomic organisation of these repeats, we have isolated a longer De1 fragment by *Xba*I digestion of *D. etrusca* genomic DNA. One of the clones obtained (p*Xba*I, containing an insert of about 8kb) was partially sequenced. A search within the EMBL/GenBank database revealed no significant similarity to any known sequence. In a terminal region of about 200 bp, p*Xba*I overlapped the *Hind*III De1 fragment (Fig. 2).

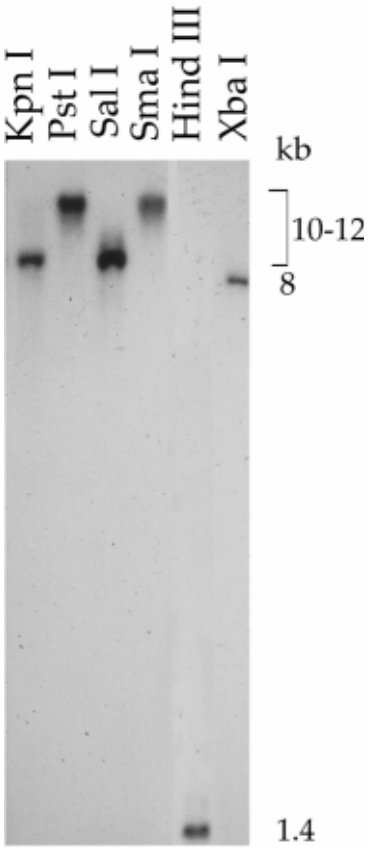

Fig. 1. – Southern blot hybridization of DIG-labelled p20 clone to *D. etrusca* genomic DNA, digested with *Kpn*I, *Pst*I, *Sal*I, *Sma*I, *Hind*III and *Xba*I restriction enzymes. *Hind*III lane shows the typical 1.4 kb band.

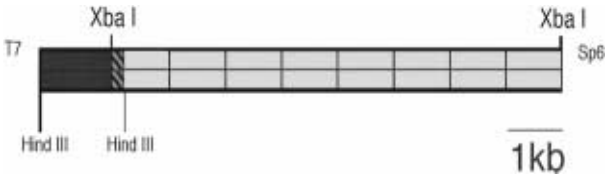

Fig. 2. – Schematic representation of De1 cloned DNA fragments. 8kb *Xba*I fragment (grey). 1.4 kb *Hind*III fragment (black). The overlapping region is shown.

The genomic organisation of De1 units was studied by analysis of the hybridization pattern revealed by p*Xba*I clone against blots of *D. etrusca* digests. The results obtained with *Hind*III, *Xba*I, *Cla*I, *Eco*RV and *Eco*RI are presented in Fig. 3. Complex patterns of hybridization bands of unrelated sizes could be observed in the different digests, suggesting that De1 elements are not organised as tandemly arranged sub-repetitions. These results are consistent with our previous observations (BATISTONI et al., 1998), and confirm that De1 DNA is made up of long repeats, scattered throughout the genome, and probably originated from transposable elements.

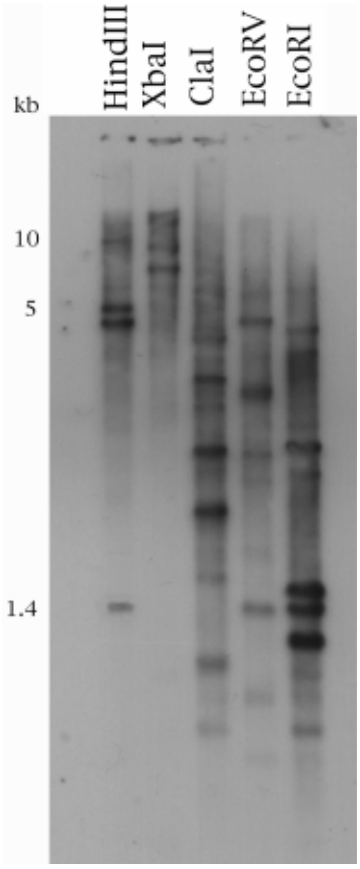

Fig. 3. – Southern blot hybridization of DIG-labelled p*Xba*I to *D. etrusca* genomic DNA, digested with *Hind*III, *Xba*I, *Cla*I, *EcoRV* and *EcoRI* restriction enzymes. The typical 1.4 kb band is present in the *Hind*III lane.

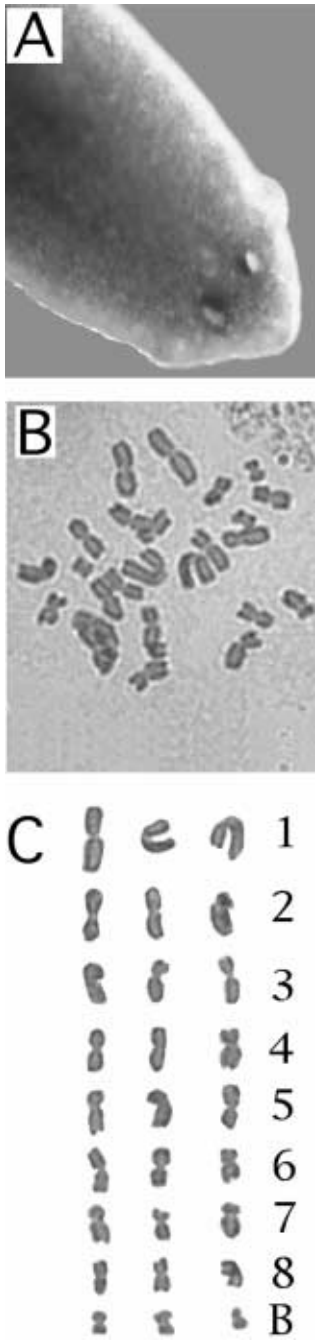

Fig. 4 (at the right). – *D. gonocephala* s.l. from Montecristo Island. (A) Dorsal view of a specimen. (B) Mitotic metaphase. (C) Triploid complement consisting of 24 chromosomes plus 3 B-chromosomes.

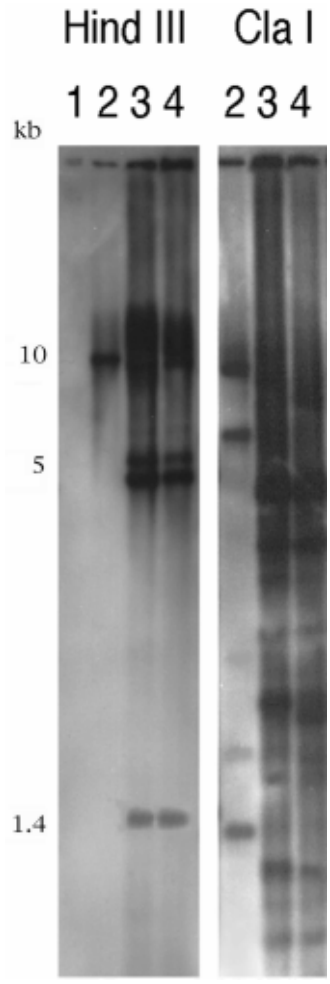

Fig. 5. – Southern blot hybridization of DIG-labelled p*Xba*I to genomic DNA from different planarians digested with *Hind*III and *Cla*I restriction enzymes. Lanes 1-4: *D. sicula*; Montecristo population, *D. etrusca* from Pisa, *D. etrusca* from Donoratico.

The use of molecular markers: the case of an asexual population of *D. gonocephala* s.l. from Montecristo Island

Some specimens of an asexual population of planarians ascribable to *D. gonocephala* s.l. (Fig 4A) were collected in a small stream on Montecristo Island. All examined specimens presented 24 standard chromosomes and 1-3 B-chromosomes, suggesting a triploid complement with respect to a haploid number n=8. However, such a complement can be more likely referred to an aneuploid condition, although numerically balanced (Fig. 4 B,C).

We used De1 DNA as a molecular marker in order to investigate the presence and the organisation of these sequences in the genome. Southern blot hybridization demonstrated that De1-like repeats are present in this population, but show differences in the restriction pattern with respect to those found in other planarians (Batistoni et al., 1998; 1999). Fig. 5 shows the hybridization pattern obtained with p*Xba*I clone in digests of planarians from Montecristo and two different populations of *D. etrusca*. No detectable signal can be observed in *D. sicula*, again confirming the specificity of De1 DNA for some species.

The genetic diversity of the specimens collected on Montecristo Island with respect to the other species that colonise both the Tyrrhenian islands and the Italian peninsula, suggests a possible specific identity of this population.

Geographic isolation probably plays a key role in speciation events of *D. gonocephala* s.l. (cf. Benazzi, 1982).

We can mention, as an example, the presence of at least eight well-identified species on the eastern Mediterranean, specifically Greece and the Greek islands (De Vries, 1984).

D. gonocephala s.l. is also well represented in the Western Mediterranean. A number of species from this geographical area was studied in detail over a long time by Benazzi and his school (cf. Mancino, 1998; Deri et al., 1999). In particular, the presence of three species was recorded on the Tuscan Archipelago: *D. ilvana* and *D. sicula*, from Elba Island (Lepori, 1948; Benazzi, 1950) and *D. benazzii* from Capraia Island (Benazzi, 1961).

RAPD analysis

In a preliminary attempt to develop a quick method to discriminate among different species and populations of *D. gonocephala* s.l., we applied a method based on PCR amplification, using single random primers of arbitrary nucleotide sequence (RAPD). This technique, often utilized as a valuable tool to determine genetic relationships in a variety of organisms, yielded interesting electrophoretic phenotypes also in planarians.

Results obtained with the primer 5'-TACCGACACC-3' showed substantial intraspecific variation in banding patterns, discriminating among different populations of *D. etrusca*. Peculiar RAPD profiles also differentiated planarians from Montecristo, as well as *D. sicula* and *D. benazzi* populations (Fig. 6).

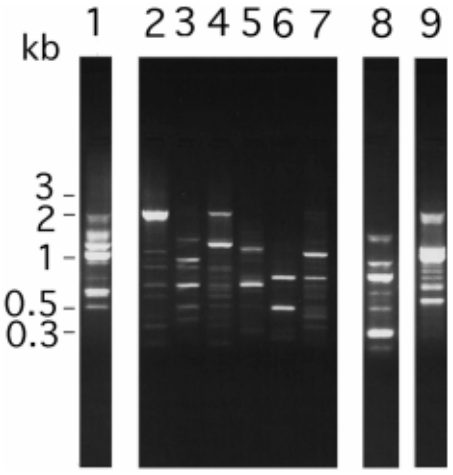

Fig. 6. – RAPD profiles of *D. gonocephala* s.l. planarians. 1: Montecristo population; 2-7: various populations of *D. etrusca* (2, Donoratico; 3, Torniella; 4, Rio di Calci; 5, Colli Berici; 6, Montemoro; 7, Revigliasco); 8: *D. sicula*; 9: *D. benazzii*.

The use of this technique with a larger number of primers should almost certainly open up the possibility of analysing genomic differences at the species, population and also individual levels. In turn this could represent a further tool for the investigation of evolutionary and biogeographical relationships in this planarian group.

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