RESEARCH NOTES

Composition and relative abundance of microsatellite repeats in genome of *Littorina saxatilis* (Olivi) (Gastropoda: Littorinidae)

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Microsatellites are di- to pentanucleotide tandem repeats, which are densely distributed throughout animal genomes.[1] Owing to their genetic and evolutionary characteristics (including high abundance in eucaryotic genomes, high level of polymorphism, inheritance in a simple Mendelian fashion and presumptive selective neutrality), microsatellites provide a large new pool of genetic markers and are widely used in population genetics studies.[1,2,3,4,5] However, the main limiting step in the use of these genetic markers is the time- and effort-consuming procedure of their isolation and characterisation. For a successful isolation of microsatellite markers it is very important to have information about abundance and length distribution of different types of repeats in the genome of an organism under study. However, despite rapidly rising interest and increasing number of studies on microsatellites, such data are as yet scarce and incomplete, and for many taxa totally absent.[6] Presently, it is known that the most abundant motifs in plants are GA and AT repeats,[7,8] and in animals, GT motifs.[9] In general, the abundance of microsatellites in the genomes of invertebrates is lower than in vertebrates.[6]

Intertidal gastropods of the genus *Littorina* are common inhabitants of rocky shores all over the world.[10] In recent decades, the genetic structure of populations and the genetic differentiation and microevolution of *Littorina* species have been extensively studied and much data on intra- and interpopulational genetic differentiation have been accumulated using polymorphic allozyme markers[11,12,13] and genetically determined shell colour morphs.[14,15] However, these genetic markers may be selectively non-neutral,[16,17,18,19] which makes it impossible to distinguish between the effects of gene flow, stochastic genetic processes and selection on the population structure of the studied species. Use of selectively neutral microsatellite markers would greatly enhance population genetic studies of these molluscs by providing a new insight into the effects of gene flow and random genetic events. However, until now microsatellites have only been characterised for two littorinid species, *Littorina striata*[20] and *Littorina subrotundata.*[21] In this communication we present data on the composition and abundance of some types of microsatellite repeats in the genome of the common North Atlantic species *Littorina saxatilis* in order to find the most promising and convenient microsatellite repeats for the future development of microsatellite markers for this and related species.

Microsatellites were isolated according to the enrichment procedure of Kijas *et al.*[22] Briefly, 5 μg of genomic DNA were digested to completion with *Sau3A* I and fragments were fractionated in 1.5% agarose gel. 300–800 bp fragments were excised from the gel, purified and ligated to SAU linkers.[23] Synthetic biotinilated oligos—$(GACA)_7$, $(CAA)_9$, $(GA)_{15}$ and $(GT)_{15}$ were obtained from MWG Biotech AG (Ebersberg , Germany). 100 ng of ligated DNA was denatured and hybridised separately to each of biotinilated oligos under the appropriate temperature (melting temperature T_m −5°C). The hybridisation mixture was incubated with Streptavidin M-280 magnetic beads (Dynal, Hamburg, Germany) according to the manufacturer's instructions. Beads with the attached fragments were washed 3 times at low stringency (2 × SSC, room temperature) and 3 times at high stringency (0.1 × SSC, 50°C). Finally, the beads were incubated in 30 μl ddH_2O at 95°C to release any single stranded DNA. 2 μl of the eluate were used in PCR with adaptor as a primer to rebuild the second strand of the fragments. After this, the enrichment procedure was repeated. 1.5 μl of gel purified product of the second enrichment were cloned using TOPO TA Cloning Kit for Sequencing (Invitrogen, Groningen, Netherlands) according to the manufacturer's protocol. Partial DNA libraries enriched for each of the 4 studied repeats (GACA, CAA, GT, GA) were equally represented in the final DNA mixture used for cloning. After cloning, 85 colonies were picked at random and sequenced manually (T7 Sequenase v.2 Sequencing Kit, Amersham Pharmacia Biotech, Freiburg, Germany).

The number of repeats and the total length of different types of microsatellites were compared by the Student's t-test with Bonferroni corrections for multiple comparisons.[24] Where necessary, the data were log-transformed to ensure normal distribution.

The enrichment procedure was found to be highly efficient in *L. saxatilis*; in the 85 bacterial clones analysed, 71 microsatellites and 2 minisatellites (with lengths of repeated units 15 and 37 bp) were found. We found 15 types of microsatellite repeats in *L. saxatilis* (Table 1), which together with the data reported for *L. striata*[20] and *L. subrotundata*[21] gives 18 types of microsatellite repeats presently known for the genus *Littorina*. Consistent with the previously reported findings for animal genomes in general,[9] the most abundant repeats in *L. saxatilis* were GT motifs (26.4%). GA and CAA repeats were also common (23.6% and 19.4% of all isolated microsatellites, respectively). In contrast, only 4.2% of all microsatellites contained GACA repeats. The observed low frequency of GACA microsatellite arrays is in agreement with previously reported findings that microsatellite motifs with longer repeat units (4 nucleotides and more) are much rarer in the genome than 2- or 3-nucleotide repeats.[9] Besides

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Table 1. Microsatellite repeat motifs found in *Littorina* species.

Type of repeat	Organism
GT, GA, GC, CAT, CAA, CCA, CGA, GACA, CATA, AGTG, CAAA, TCAA, CGCA, AACAAG, AAAGTCC	*L. saxatilis* This study
GT, GA, GACA	*L. striata*[20]
ATA, AAT, CTT, TCAA, CAA, GT, GA	*L. subrotundata*[21]

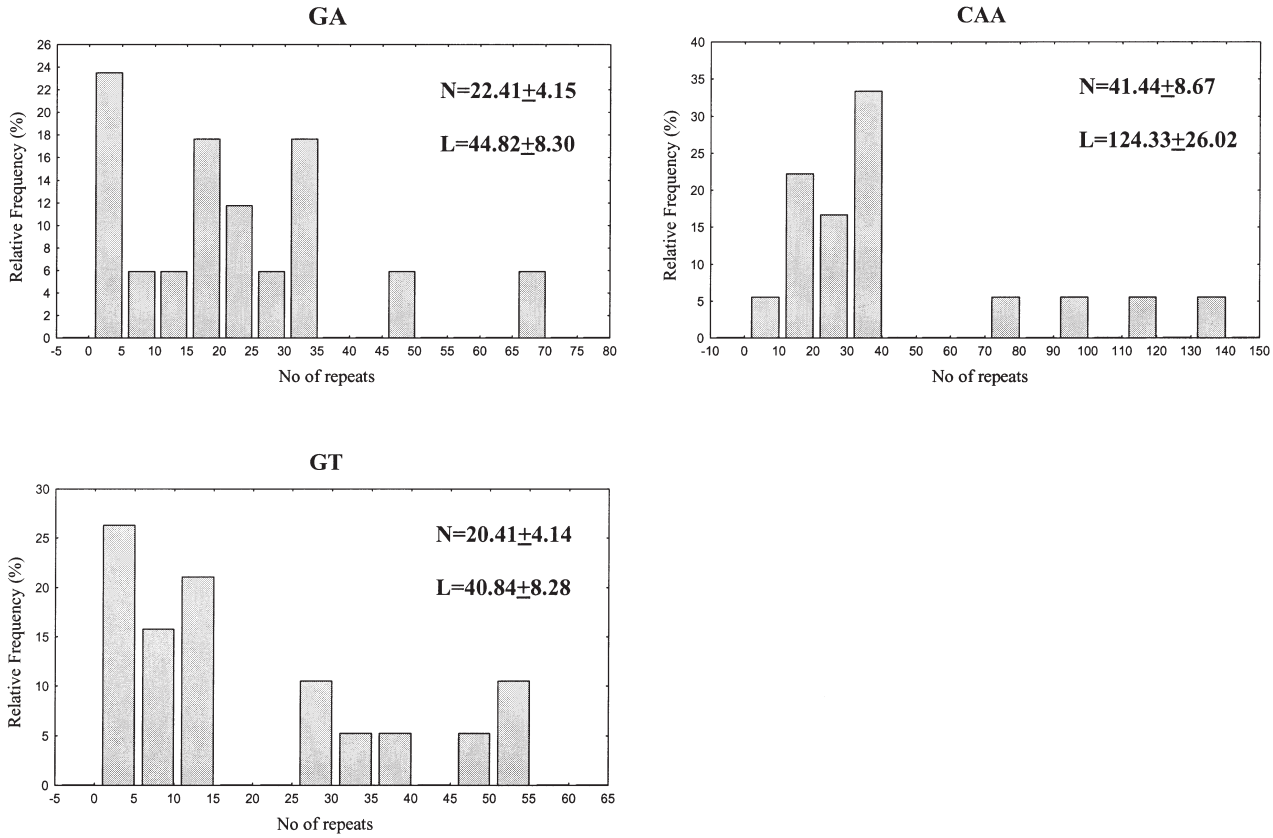

Figure 1. Length distribution of different types of microsatellites (GA, GT and CAA repeats) in the genome of *L. saxatilis.* Horizontal axis—length classes (number of repeats), vertical axis—relative frequency of microsatellites with the given number of repeats (%). Average number of repeats (N) and average length of microsatellite (L, base pairs) (± standard error) are given for each motif type.

the motifs for which the genomic library was enriched (GACA, CAA, GT, GA), we also found CCA repeats (5.6%), CGA (4.2%), CATA (2.8%), CAAA (2.8%), and one array of each AACAAG, GC, CAT, TCAA, CGCA, AGTG and AAAGTCC repeats. 32 microsatellites (45%) contained perfect repeats and 39 (55%) interrupted repeats. However, it should be noted that the absolute frequencies of different types of repeats in the enriched genomic library may not necessarily be representative of the relative abundance of different microsatellite repeats in the genome of *L. saxatilis*. It has been shown that the probability of finding certain nucleotide repeats may also depend on the restriction endonuclease used for the digestion of the genomic DNA for a partial genomic library.[25] A less biased composition of different repeat types could be obtained by replacing the endonuclease digestion with sonication.[26]

Length distribution differed between the three commonest types of microsatellites found in this study (Fig. 1). Among GA and GT repeats, relatively short arrays (< 20 repeats) were most abundant, while among the CAA microsatellites most arrays contained 30 to 40 repeats. The average number of repeats did not differ significantly between GA and GT types or between GA and CAA microsatellites ($p > 0.10$) (Fig. 1). However, GT microsatellite arrays consisted on average of less repeats than CAA microsatellites ($p < 0.01$) (Fig. 1). At the same time the total length of CAA microsatel-

lites was significantly higher than in arrays of GA and GT types ($p < 0.01$) (Fig. 1).

The data on the composition and length distribution of microsatellites obtained in the present study can be used for choosing the optimal repeat motifs for microsatellite isolation in other *Littorina* species and in closely related genera. One of the important problems of microsatellite analyses is the reduction of the so-called stutter bands. Individual microsatellite alleles often do not appear as a single discrete product, but rather as a series of bands descending in size and intensity from the main product. These additional bands are referred to as stutter or shadow bands. It has been shown that longer repeats (4 base pairs) tend to stutter less than shorter (3 or 2 base pair motifs),[27] and longer repeat sequence arrays increase stutter band intensity.[28] In the partially enriched library used in this study, CAA repeats were well represented (nearly 20% of all isolated microsatellites), and they were more convenient for PCR screening than GA and GT motifs because of less stuttering (Sokolov, unpublished data). Hence, in the case of *Littorina* species a good compromise between the time invested in the isolation of highly polymorphic microsatellite loci and their convenience for subsequent PCR screening would be the use of CAA motifs.

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