Vol. 203, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 30, 1994 Pages 646-651

DIRECT TYPING OF POLYMORPHIC MICROSATELLITES IN THE COLONIAL TUNICATE *Botryllus schlosseri* (ASCIDIACEA)

Zeev Pancer[1, 2], Harriet Gershon[2] and Baruch Rinkevich[1]

[1] Israel Oceanographic and Limnological Research, National Institute of Oceanography, Dept. of Marine Biology, POB 8030, Haifa 31080, Israel

[2] Technion-Israel Institute of Technology, Bruce Rappaport Faculty of Medicine, Dept. of Immunology, POB 9649, Haifa 31096, Israel

Received July 16, 1994

Summary. Five microsatellite loci of the marine protochordate *Botryllus schlosseri* were cloned: four of uninterrupted $(AG)_n$ repeats and one of both $(AG)_n$ and $(TG)_n$ repeats. By means of an innovative procedure small colony fragments were minimally treated to serve as templates for PCR with microsatellite-specific primers. Four of the loci were polymorphic: 7-8 discrete alleles were scored in nine colonies, heterozygosity ranging between 44-80%. At locus number 811 spacing of the alleles and gel-resolution were highest, therefore, ten additional colonies were typed and in total nine alleles were scored with maximal allelic interval of 120 base pair and 53% heterozygous colonies. The high levels of microsatellite-polymorphism provide a new tool as individual markers for studies on aspects of the botryllid polymorphic allorecognition system.

The compound ascidian *Botryllus schlosseri* is a sedentary marine-invertebrate, abundantly inhabiting shallow waters throughout the world. Colonies consist of from several to hundreds of individual zooides, arranged in typical rosette-shaped structures, that are embedded in a transparent gelatinous tunic. The colony shares a ramified vascular network of circulating hemolymph and blood cells. Botryllid ascidians are commonly used as model systems for studies of allorecognition in invertebrates and the evolution of vertebrate tissue histocompatibility due to a unique fusibility/histocompatibility (Fu/HC) system (reviewed in refs. 1, 2), originally described 90 years ago in *B. schlosseri* (3). Histocompatibility discrimination is controlled by a single highly polymorphic locus, with codominantly expressed Mendelian haplotypes; recognition is manifested by the simple rule that colonies whose growing edges meet, may either fuse their vasculature within hours of contact if they share one or both alleles at the locus, whereas mismatched allelic combinations lead to conspicuous allogeneic rejection (4-6). It is estimated that up to 100 different Fu/HC alleles exist in the population (7, 8).

In order to study parameters of the allorecognition system of *B. schlosseri* we were in need of polymorphic markers, that will enable individual identification of genotypes even from

0006-291X/94 $5.00

minute samples. PCR amplification of microsatellite-loci, those tandem repeats of 1-6 nucleotides so abundant in eukaryotic genome, that reveal a high level of allelic polymorphism in the number of motif-repeats (9-12), seemed appropriate. Microsatellite amplification products provide powerful molecular markers for individual identification, population studies, linkage analysis and genetic mapping (reviewed in refs. 13, 14). Microsatellite-typing has been applied in a variety of species. Among vertebrates, primarily in mammals, and recently also in chicken (15) and fish (16, 17). However, invertebrate reports of microsatellite-typing are limited to insects (11, 18-20).

Here we describe the cloning of five microsatellite-loci from *B. schlosseri* and a new method for direct PCR-typing of these loci from tissue subjected to minimal preparative steps, applied on east-Mediterranean (Israel) and Pacific (California, USA) colonies.

Materials and Methods

Animals. Tunicates were maricultured in the laboratory as previously described (21). Mediterranean-sea colonies were collected from Tel-Shikmona, in the vicinity of the National Institute of Oceanography, Haifa, Israel. Pacific-sea colonies were from our laboratory stock which originated from Monterey Marina, CA, USA (21).

Materials. Molecular biology reagents were obtained from Boehringer Mannheim and Sigma; restriction endonucleases, modification enzymes and random primed DNA labeling kit from Boehringer Mannheim; Lambda gt10 and Bluescript KS^+ from Stratagene; Hybond N 82 mm and 132 mm hybridization membranes and [α-^{32}P]dCTP from Amersham; Sequenase Version 2 sequencing kit from USB.

Cloning of microsatellites. High molecular weight DNA was isolated by organic deproteination (22), and then extraction with CTAB (hexadecetyltrimethylammoniumbromide) as follows: The DNA was resuspended in 0.5 ml CTAB buffer (0.15 M NaCl, 0.1 M Tris pH=8, 0.1 M EDTA, 2% CTAB, 0.2% β-mercaptoethanol, 0.4 mg/ml Proteinase K) and incubated 1 h at 65°C, then extracted with CIA (chloroform: isoamylalcohol, 24:1) and then with phenol:CIA (25:24:1). A small-insert genomic library (length averaged 1 kb) in Lambda gt10 was constructed from a pool of 10 Mediterranean-sea colonies as described (23), corresponding to $1.5x10^7$ pfu/ml. 10^5 pfu were screened by hybridization to one of the two ^{32}P-labeled microsatellite probes, one of 160 bp (24): 5'$(GACA)_7(GA)_{28}(GT)_6TT(GT)_6C(GT)_8C(TGTC)_8$3'; the second of 217 bp: 5'$(GACA)_5$ $(GGGAGACA)_2[(GA)_3CA]_{10}(GGGAGACA)_2[(GA)_3CA]_4ACAGATAG[AC(AG)_3]_4AC(AG)_{21}AC(AG)_6(TG)_4TT$ $T(GT)_3ATG(TGTC)_5$3'. Both probes were cloned from random PCR-amplifications of *B. schlosseri* genomic DNA with the single primer $(GACA)_5$. Hybridization conditions were as recommended by the filters manufacturer. Positive plaques were purified by an additional screening cycle, subcloned and sequenced (data in Table 1).

PCR reactions. Colony samples for typing with microsatellite loci-specific primers consisted of one to five zooids that were teased off the tunic under a dissection microscope. Tissue samples were transferred into 1.5 ml test tubes and were boiled for 5 min in 100 µl TE, dispersed by pipetation through an aerosol free tip (ELKAY, Labsystems) and centrifuged at 12K g for 5 min. The supernatant was discarded, the pellet resuspended in 10-20 µl of PCR-lysis buffer (20 mM DTT, 10^{-3}% SDS and 0.5 mg/ml Proteinase K) and incubated for 1 h at 55°C. The reaction was stopped by boiling again for 5 min. Amplifications were performed in 10 µl reaction mixtures containing 1 µl of the sample, 4 pmoles each of the forward and reverse primers (see data in Table 2), 0.2 mM of each dNTP, 0.1 µCi [α-^{32}P]dCTP, 5% DMSO, 0.5 U *Taq* DNA polymerase and buffer, supplemented to 2 mM Mg^{++} for all the microsatellite amplifications except for locus

Table 1. Features of five *B. schlosseri* microsatellite clones

Clone	Accession	Repeat sequence
Bsg.321	X77906	$(AG)_{19}$
Bsg.531	X77907	$(AG)_{10}A(AG)_4A(AG)_4T(AG)_2AA(TG)_7$
Bsg.711	X77904	$(AG)_{15}$
Bsg.721	X77905	$(AG)_{40}$
Bsg.811	X77903	$(AG)_{40}$

811. The cycling parameters (PTC-100, MJ Research) are summarized in Table 2. Reactions were stopped by the addition of 5 µl Sequenase stop solution (USB), denatured for 5 min at 95°C, then 2 µl of each sample were electrophoresed on a 6% denaturing polyacrylamide gel. A sequencing ladder served as a size marker. Allele length was determined from the longest of the strong bands, and colonies were scored as heterozygous only if the alleles differed by more than two bp.

Results and Discussion

The small-insert genomic DNA was screened with two oligonucleotide probes: $(GTG)_7$ and $(GACA)_5$, with no positive results so, instead, we used two microsatellite-probes. Five microsatellite clones were sequenced (Table 1), ranging in length 178-888 bp, all containing the basic repeat unit of $(AG)_n$. Four of the microsatellite clones had uninterrupted runs of AG, and one that was compound (Bsg.531) had several interruptions and an additional TG motif. Of the five clones Bsg.321 and Bsg.531 were detected with the 160 bp probe, and the other three with the 217 bp probe. Previously we used the 160 bp probe to screen a *B. schlosseri* cDNA library,

Table 2. PCR primers for five microsatellite loci and the corresponding cycling parameters

Primer code and sequence (5'-3')	incubation temperature, time and number of PCR cycles
321F CGTTGCCAGAGATTATTGTTTT 321R CTATGAAGGCCGTATCGCAT	96°C 180sec: x1; 96°C 45sec, 55°C 60sec, 74°C 30sec: x35
531F CTGATGAGTAATGCCAGCACA 531R AAAACAAAAGACCTGGTGATCG	96°C 180sec: x1; 95°C 45sec, 58°C 60sec, 74°C 30sec: x35
711F CAATGTTTTGGCTGTCGGAT 711R TGCCGTGTGTTATGGATTTG	96°C 180sec: x1; 96°C 45sec, 55°C 60sec, 74°C 30sec: x35
721F TGACGGGCACAATTCATAGA 721R CCTGCATGTCGCATTACTGT	96°C 180sec: x1; 96°C 45sec, 55°C 60sec, 74°C 30sec: x35
811F TCAACTCGATGGATATGGACT 811R CGACATTGTGGGCGAGTAC	96°C 180sec: x1; 95°C 45sec, 60°C 75sec, 74°C 15sec: x30

and analyzed four positive clones, each with a truncated $(GA)_{14-54}$ microsatellite at the 5' end (accessions: X77810, X77811, X77812, and ref. 24). Hence, a probe of 28 GA repeats and a total of 20 GT repeats detected five pure GA containing clones (Bsg.321 and four cDNA clones), but only one clone that consisted of a compound-microsatellite (25) with both GA and GT repeats (Bsg.531). This may indicate that GA motifs in the genome of this protochordate outnumber the GT motifs, similar to what has been reported for bees (19), but unlike reports concerning vertebrates (15-16) and *Drosophila* (26).

The small size of the average *B. schlosseri* colony (less than 1 cm^2, and approximately 100 mg of wet weight) and especially of the embryos and larvae (less than 0.5 mm in diameter), forced us to seek an alternative way of sampling other than the DNA extraction procedure. Samples consisting of a tiny colony fragment (single zooid, gonad, embryo) or 100-1000 hemolymph cells were boiled in TE for five minutes to denature the protein and DNA, then the supernatant was discarded to eliminate soluble comtaminantes aboundant in ascidian tissues (27). The pellet was treated with a lysis buffer (modified from ref. 28) to solubelize the DNA. This treatment resulted in a sample suitable for PCR, and provided sufficient material for at least five reactions with microsatellite-specific primers (Fig. 1). Recently, a technique for direct PCR from solid tissues was published (29), based on Formamide Low Temperature (FLoT) PCR with *Tth* DNA polymerase. We did not test the FLoT technique, which consists of less procedural steps than ours but may not be effective when inhibitory contaminants must be eliminated.

B. schlosseri microsatellite-loci exhibited high levels of polymorphism at four of the five loci for both Israeli (Tel-Shikmona) and American (Monterey) colonies, with 7-9 distinct alleles and 44-80% heterozygous colonies (Table 3), comparable to recent reports (16, 17, 19, 30). The

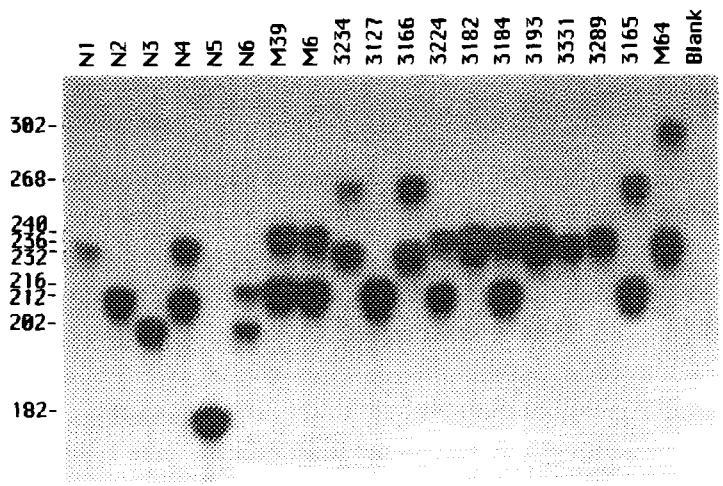

Figure 1. Typing of *Botryllus schlosseri* colonies from Israel and USA with locus 811 specific-primers. Colony samples were minimally treated by boiling and lysis buffer and served as templates for PCR amplification. Twenty reactions were separated on a 6% sequencing gel; the first six (N1-N6) were from Tel-Shikmona (Israel) and thirteen from Monterey (USA). In the last Blank lane the sample contained no DNA. Numbers at the left represent length in base pairs, measured at the highest point of the allele. In sample N6, which is slightly underexposed, it is evident that each "allele" consists of several bands, each two base pairs shorter than the previous.

Table 3. Typing *B. schlosseri* colonies from Israel (N1-N6) and USA (the rest)

Colony code	Length of microsatellite alleles at five loci (in bp)				
	811[a]	721	711	531	321
N1	232	167	82	nd[b]	159, 125
N2	212	169	96	nd	165, 159
N3	202	nd	96	nd	161
N4	236, 212	167	96	nd	161
N5	182	nd	106, 96	241	169, 163
N6	212, 202	169, 165	106, 102	234, 213	163, 125
M39	240, 216	nd	88	197, 167	157, 125
M6	240, 216	nd	88, 84	169	125
3234	268, 232	nd	110, 88	251, 223	159, 125
3127	216				
3166	268, 232				
3224	240, 216				
3182	240				
3184	240, 216				
3193	240				
3331	236				
3289	240				
3165	268, 216				
M64	302, 236				
Alleles:	9	3	7	8	7
Heterozygotes[c]:	10/19 (53)	1/4 (25)	4/9 (44)	3/5 (80)	6/9 (66)
Max. interval[d]:	120	4	28	82	44

[a] For locus 811, typing of 10 additional American colonies was performed.
[b] nd = not determined due to repeatedly unsuccessful amplifications.
[c] Numbers in parentheses are the percent heterozygotes.
[d] The length-difference between the longest and shortest alleles.

fact that there were no amplification products in some of the nine samples at loci 321 and 721 can not be attributed to our direct sampling method since aliquots from the same samples were amplified successfully with the rest of the loci specific-primers. Typing of the first nine colonies with 811 specific-primers revealed a dispersed, easily resolved distribution of eight alleles. Although only a few Israeli colonies were sampled, it is intriguing that five different alleles were scored at locus 811 in the six colonies, four of them homozygous. Ten additional American colonies were typed at this locus and 62% (8/13) were heterozygous (Fig. 1, Table 3). Such a high level of heterozygosity was an unexpected result because these colonies came from our laboratory stock, collected in Monterey Marina (CA, USA) about a year earlier (four to five average generations) and maintained in aquaria of 10-30 colonies (21). The small-population husbandry might explain the reoccurrence of the same alleles in more than one American colony, as in the case of alleles 240 and 216, found in seven and six of the colonies, respectively (Table 3). In total, nine alleles were scored at locus 811, with maximal allelic interval of 120 bp, and 53% (10/19) heterozygous colonies (Table 3).

Protochordates deserve special attention due to their phylogenetic position as the possible progenitors of vertebrates (31), and the unique polymorphic allorecognition system of botryllid ascidians. The application of molecular biology techniques and especially the advent of PCR will pave the way to study new aspects of the 90-year-old enigma of *Botryllus schlosseri*.

Acknowledgments

We wish to thank Prof. B. Z. Cavari for a critical review, R. Pancer for editing the manuscript and E. Tal for graphic assistance. This study was supported by a career Development Award to B. R. from the Israel Cancer Research Foundation-US, a grant from the US-Israel Binational Science Foundation, and a grant from S. Price, NAF/IOLR.

References

1. Weissman, I.L., Saito, Y. and Rinkevich, B. (1990) Immunol. Rev. 113, 227-241.
2. Rinkevich, B. (1992) Anim. Biol. 1, 17-28.
3. Bancroft, F.W. (1903) Proc. Calif. Acad. Sci. 3rd ser. 3, 137-186.
4. Oka, K. and Watanabe, H. (1960) Bull. Mar. Biol. Stn. Asamushi 10, 153-155.
5. Sabbadin, A. (1962) Rend. Accad. Lincei 32, 1031-1035.
6. Scofield, V.L., Schlumpberger, J.M., West, L.A. and Weissman, I.L. (1982) Nature 295, 499-502.
7. Karakashian, S. and Milkman, R. (1967) Biol. Bull. 133, 473.
8. Grosberg, R.K. and Quinn, J.F. (1986) Nature 322, 456-459.
9. Litt, M. and Luty, J.A. (1989) Am. J. Hum. Genet. 44, 397-401.
10. Smeets, H.J.M., Brunner, H.G., Ropers, H.H. and Wieringa, B. (1989) Hum. Genet. 83, 245-251.
11. Tautz, D. (1989) Nucleic Acids Res. 17, 6463-6471.
12. Weber, J.L. and May, P. E. (1989) Am. J. Hum. Genet. 44, 388-396.
13. Hearne, C.M., Ghosh, S. and Todd, J.A. (1992) TIGS 8, 288-294.
14. Queller, D.C., Strassmann, J.E. and Hughes, C.R. (1993) TREE 8, 285-288.
15. Moran, C. (1993) J. Hered. 84, 274-28.
16. Estoup, A., Presa, P., Kreig, F., Vaiman, D. and Guyomard, R. (1993) Heredity 71, 488-496.
17. Rico, C., Zadworny, D., Kuhelein, C. and Fitzgerald, G.J. (1993) Mol. Ecol. 2, 271-272.
18. Traut, D., Epplen, J.T., Weichernhan, D. and Rohwedel, J. (1992) Genome 35, 659-666.
19. Estoup, A., Solignac, M. and Cornuet, J-M. (1993) Nucleic Acids Res. 21, 1427-1431.
20. Hughes, C.R. and Queller, D.C. (1993) Mol. Ecol. 2, 131-137.
21. Rinkevich, B., Shapira, M., Weissman, I.L. and Saito, Y. (1992) Zool. Sci. 9, 989-994.
22. Graham, D.E. (1978) Anal. Biochem. 85, 609-613.
23. Kandpal, R.P., Kandpal, G. and Weissman, S.M. (1994) Proc. Natl. Acad. Sci. USA 91, 88-92.
24. Pancer, Z., Gershon, H. and Rinkevich, B. (1993) Bichem. Biophys. Res. Comm. 197, 973-977.
25. Weber, J.L. (1990) Genomics 7, 524-530.
26. Tautz, D. and Renz, M. (1984) Nucleic Acids Res. 12, 4127-4138.
27. Kumar, S., Degnan, B.M., Ross, I.L., Hawkins, C.J. and Lavin, M.F. (1988) Marine Biol. 98, 95-100.
28. Gyllensten, U. (1990) In PCR Protocols. A Guide to Methods and Applications (M.A. Innis, D.H. Gelfand, J.J. Sninsky and T.J. White, Eds), pp 300-306. Academic Press, San Diego, CA.
29. Panaccio, M., Georgesz, M., Hollywell, C. and Lew, A. (1993) Nucleic Acids Res. 21, 4656.
30. Lagercrantz, U., Ellegren, H. and Andersson, L. (1993) Nucleic Acids Res. 21, 1111-1115.
31. Berrill, N.J. (1955) The origin of vertebrates. Oxford Univ. Press, New York.