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CLONING AND SCREENING OF DNA PROBES FOR GENETIC STUDIES IN STONE CRABS (DECAPODA: XANTHIDAE: *MENIPPE*)

Joseph E. Neigel, Darryl L. Felder, Caryl A. Chlan, and Rachel La Porte

ABSTRACT

A recombinant library was constructed from genomic DNA of *Menippe adina* and characterized by Southern blot analysis. Cesium chloride/ethidium bromide equilibrium centrifugation of testes lysates yielded high molecular weight DNA of sufficient purity for restriction endonuclease digestion. The library was constructed in the plasmid pUC19 from genomic DNA of *Menippe* that had been partially digested with the restriction endonuclease Sau3A I. A Southern blot of nine randomly chosen clones from this library was hybridized with total genomic DNA to assess relative abundances of these sequences in the genomes of *M. adina* and *M. mercenaria*. These assessments were consistent with the results of genomic Southern blot analysis in which specific clones were used as hybridization probes on blots of total genomic DNA. Potential applications of these cloned sequences as hybridization probes for evolutionary genetic studies are discussed.

Rapid and inexpensive methods for the characterization of DNA sequence variation are needed to extend the investigation of DNA level polymorphisms to a diverse array of organisms. Single nucleotide substitutions, small rearrangements, and variation in the length of tandem arrays of repeated sequences have been used in the analysis of a variety of population genetic and systematic problems (Avise *et al.*, 1987; Burke and Bruford, 1987; Hallerman and Beckmann, 1988). At present, the technique of Southern hybridization (Southern, 1975) provides the most general and efficient means to detect nuclear sequence variation. However, application of Southern analysis to most crustaceans is impeded by lack of cloned nucleic acid probes of appropriate homology. Studies of cloned DNA sequences in the Crustacea have focused primarily on satellite repetitive sequences (e.g., Bonnewell *et al.*, 1983; Fowler *et al.*, 1985; Cruces *et al.*, 1986), and on sequences from *Artemia* (van Hemert *et al.*, 1983; Cruz-Alvarez and Pellicer, 1987; Vaughn *et al.*, 1984; Maassen *et al.*, 1985; Andrews *et al.*, 1987).

Although probes cloned from unrelated species may be suitable if they contain highly conserved sequences, such sequences may exhibit little variation within populations or among closely related species. Alternatively, hybridization probes may be cloned directly from the species to be studied (e.g., Quinn and White, 1987), and this approach has been taken in our study of the stone crabs *Menippe mercenaria* (Say, 1818) and *M. adina* (Williams and Felder, 1986). Relatively simple methods of DNA extraction and genomic library construction were found to yield a large number of potentially useful hybridization probes. Herein we report on how these methods were applied to *Menippe*, and present an initial characterization of nine hybridization probes.

MATERIALS AND METHODS

DNA Isolation and Purification

Live specimens of *Menippe mercenaria* were obtained from Naples, Florida, and of *M. adina* from Cocodrie, Louisiana. Whole DNA was extracted from the testes of crabs by a procedure similar to that described by Strauss (1987) for mammalian tissue. Between 1 and 5 g of testicular tissue was frozen in liquid nitrogen in a prechilled mortar and ground to a fine powder. Additional liquid nitrogen was added to the mortar during grinding to prevent the tissue from thawing. The frozen powder was transferred to 1.2 volumes of lysis buffer (100 mM NaCl, 10 mM Tris pH 8, 25 mM EDTA, 0.5% SDS, 0.1 mg/ml proteinase K (Sigma Chemical Co., P.O. Box 14508, St. Louis, Missouri 63178)) at 50°C. This suspension was incubated overnight at 50°C with shaking at 100 oscillations per min, and was followed with 1 of 2 purification methods. The first method included phenol extraction to remove proteins. Each lysate was mixed with an equal volume of a 25:24:1 mixture of buffered phenol, chloroform, and isoamyl alcohol by gentle shaking in a 40-ml polypropylene Oak Ridge tube and centrifuged at 3,000 × *g* for 15 min. The aqueous phase (top layer) was removed and reextracted 3–5 times until it became clear. A final

extraction with an equal volume of a 24:1 mixture of chloroform and isoamyl alcohol removed traces of phenol. DNA was precipitated by addition of one-half volume of 7.5 M ammonium acetate and 2.5 volumes of 100% ethanol at room temperature. The DNA was pelleted by centrifugation at 5,000 × *g* for 5 min, dried under vacuum, and resuspended in 1 ml of 10 mM Tris pH 8.0, 1 mM EDTA (TE). DNA samples were purified by CsCl density gradient centrifugation as described below.

In the second method, DNA was purified directly from lysates by cesium gradient centrifugation. Solid material in the lysate was allowed to settle for 15 min at room temperature. Then, 5.4 g of finely ground CsCl and 40 μl of 10 mg/ml ethidium bromide in TE was mixed with 5.5 ml of the lysate. If the CsCl did not dissolve by gentle rocking after 1 min, the solution was heated to 50°C to enhance solubility.

Dye Buoyant Density Gradient Centrifugation of Genomic DNA Samples

After the addition of CsCl and ethidium bromide, the density of the solution was checked by weighing 100 μl aliquots and adjusted to between 1.56 and 1.58 g/ml. Samples were then transferred into Beckman ½ × 2″ polyallomer quick seal tubes and centrifuged at 50,000 rpm (250,000 × *g*) for 12–16 h in a Beckman Vti65.2 vertical rotor. Following centrifugation, DNA bands were generally visible in room light. Occasionally, UV illumination (366 nm) was needed to visualize small yields of DNA. Bands were removed with an 18-gauge needle hypodermic syringe. A second centrifugation under the same conditions was used for further purification when necessary.

Ethidium bromide was removed by repeated extraction with equal volumes of cesium-saturated isopropanol. After the aqueous phase became colorless, CsCl was removed by dialysis against 2 l of 10 mM Tris, pH 8, 10 mM NaCl, 0.1 mM EDTA with several changes of buffer over a 2-day period. After dialysis, DNA concentrations were determined by absorbance measurements.

Cloning Hybridization Probes

Standard cloning methods (Maniatis *et al.*, 1982; Ausubel *et al.*, 1987) were used to construct a library of genomic fragments in pUC19. Genomic DNA samples were partially digested with Sau3A I to yield fragments from 1–5 kb in size. Digestion conditions were determined empirically, with the extent of digestion assessed on ethidium-stained 0.7% agarose gels. pUC19 was linearized by digestion with BamH I and treated with calf alkaline phosphatase (Boehringer Mannheim Corp., P.O. Box 50528, Indianapolis, Indiana 46250) to prevent self-ligation. Genomic fragments were ligated into the BamH I site with T4 DNA ligase (United States Biochemical Corp., Dept. T, P.O. Box 22400, Cleveland, Ohio 44122), and used to transform competent *E. coli* (strain JM83) cells. Ampicillin resistant, β-galactosidase minus colonies were identified on L-Agar plates with 50 μg/ml ampicillin (Sigma) to which were added 40 μl of a 2% solution of 5-bromo-4-chloro-3-indolyl-B-D-galactoside (X-gal, Sigma) dissolved in dimethyl formamide and 40 μl of a 100 mM solution of isopropyl-B-D-thiogalactopyranoside (IPTG, United States Biochemicals), and cultured. One hundred colonies were used to inoculate 5-ml cultures, which were grown overnight in L-Broth with 50 μg/ml ampicillin, and then stored as frozen glycerol stocks (Maniatis *et al.*, 1982). Nine putative recombinants were selected at random to produce 100-ml cultures from which plasmid DNA was isolated by alkaline lysis (Maniatis *et al.*, 1982) and purified by cesium gradient centrifugation, as described above.

Southern Blot Analysis

Genomic DNA samples were prepared for Southern (1975) blot analysis as follows. Five-μg samples of genomic DNA were digested by restriction endonucleases according to the supplier's directions. If the volume of a digest exceeded 15 μl, it was concentrated by ethanol precipitation after completion of the digestion. Samples were brought to a final volume of 15 μl with TE and mixed with 2 μl loading buffer. Samples were electrophoresed in 0.7% agarose gels with 1× TBE (0.089 M Tris-borate, 0.089 M boric acid, 0.01 M EDTA, pH 8.0) at 6 V cm^{-1} for 5 h, stained with 0.5 μg/ml ethidium bromide and photographed.

Gels were blotted (Southern, 1975) as follows. DNA was denatured by immersion of the gel in at least 3 volumes of 0.25 M NaOH and 1 M NaCl for 30 min with 1 change of solution. The gel was then neutralized by immersion in several volumes of 0.5 M Tris, pH 7.5, 1.5 M NaCl for 30 min with 1 change of solution. DNA was transferred to Magnagraph® nylon membranes (Micron Separations, Inc., 135 Flanders Rd., P.O. Box N, Westborough, Massachusetts 01581) by capillary blotting with 10× SSC (1.5 M NaCl, 0.15 M sodium citrate), washed briefly in 5× SSC, and baked at 80°C for 1 h.

Random primed DNA labeling of probes with digoxigenin-dUTP, hybridization of nylon membranes, and immunological detection of probes were performed with the Boehringer Mannheim "Genius DNA Labeling and Detection Kit" according to the supplier's directions. Probe DNA was labeled with the hapten digoxigenin by random primer extension with DNA polymerase I Klenow fragment (Ausubel *et al.*, 1987). Hybridizations were performed overnight at 65°C in 5× SSC and 10–50-ng probe per ml hybridization solution. The bound probe was visualized by incubation with polyclonal sheep anti-digoxigenin Fab fragments conjugated to alkaline phosphatase, which produces a localized color reaction when incubated with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate toluidinium.

Results

Isolation and Purification of Genomic DNA

Several grams of testes were dissected from each adult stone crab. DNA yields per individual averaged 5–10 mg. In contrast, extractions of DNA from other tissues, which generally contain fibrous material or pigments, required more extensive isolation and purification procedures and produced lower yields (data not shown).

Two methods of DNA purification were

compared. One method included phenol extraction to remove proteins from crude lysates, the other used one or two repetitions of cesium gradient centrifugation to remove contaminants. While both methods yielded substantial quantities of DNA, phenol extraction appeared to result in considerable shearing or degradation of the DNA, as assessed by agarose gel electrophoresis. Without phenol extraction, DNA of high molecular weight was routinely obtained (Fig. 1, compare genomic DNA in lane 4 with marker DNA in lane 5). Furthermore, as shown in Fig. 1, genomic DNA purified without phenol extraction did not appear to inhibit the activities of restriction endonucleases or to migrate abnormally in agarose gel electrophoresis. Lane 3 shows that a 0.5-μg aliquot of high molecular weight genomic DNA, equivalent to that in lane 4, appears to be completely digested by EcoR I. As an additional test for the presence of contaminants that would inhibit digestion, 0.5 μg of bacteriophage λ DNA was added to a third aliquot of genomic DNA of *Menippe* and incubated in this mixture with EcoR I. The products of this digestion were run in lane 2. Lane 1 shows a control EcoR I digestion of λ alone. A comparison of lanes 1 and 2 confirms that the λ DNA was digested completely and electrophoresed normally in the presence of the genomic DNA preparation of *Menippe*.

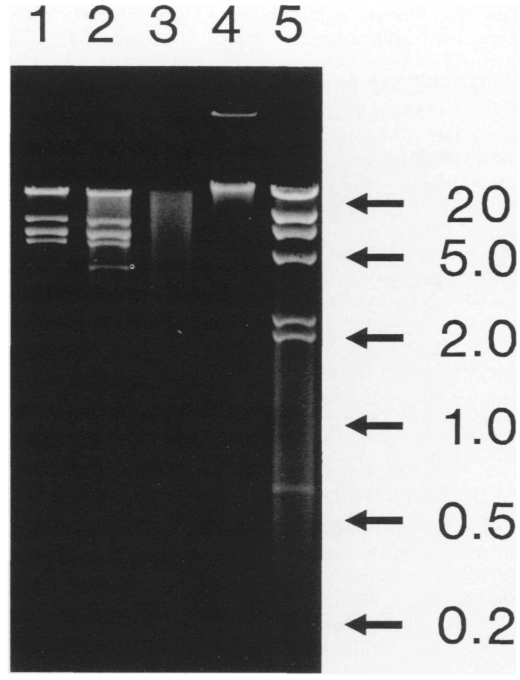

Fig. 1. Agarose gel electrophoresis of DNA of *Menippe* isolated from testes. Ethidium bromide flourescence after electrophoresis through a 0.7% agarose gel for 6 h at 5 V cm^{-1}. Lane (1), EcoR I digest of 0.5 μg bacteriophage λ DNA; (2) EcoR I digest of 0.5 μg genomic DNA of *M. adina* mixed with 0.5 μg phage λ DNA; (3) EcoR I digest of 0.5 μg genomic DNA of *M. adina* alone; (4) 0.5 μg undigested genomic DNA of *M. adina* incubated 6 h at 37°C with EcoR I buffer; (5) size standard of bacteriophage λ DNA digested with Hind III.

Assessment of Copy Number

The extent to which randomly cloned DNA sequences are repeated within a genome of *Menippe* was assessed in two ways. First, Southern blots of the nine cloned sequences were probed with labeled total genomic DNA from *Menippe adina.* The amount of labeled DNA that hybridized to each cloned sequence should reflect the number of copies of homologous sequences in the genome. This rationale was used by Wichman and co-workers (1985) to detect phylogenetic differences in copy number for cloned sequences, who termed the method ϕ-screen (ϕ for phylogenetic). Figure 2 shows an electrophoretic separation of restriction fragments for nine clones that had been digested by combinations of restriction endonucleases that yield a 2.7 kb plasmid vector fragment, and one or more additional fragments from the DNA insert of *Menippe.* Amounts of DNA loaded for each clone were adjusted to yield approximately 200–500 ng DNA per fragment for fragments in the 0.5–2-kb size range.

A Southern blot of the gel shown in Fig. 2, probed with digoxigenin-labeled genomic DNA of *Menippe adina,* is shown in Fig. 3A. The 2.7-kb pUC19 fragment and the fragments of λ DNA used as size standards allow the extent of nonspecific hybridization to be assessed. Low levels of hybridization to pUC19 were detectable after prolonged development of the tetrazolium color reaction, and then only if amounts in excess of 500 ng DNA were present. The faint and diffuse hybridization in lane 1 does not correspond to the fragments of λ DNA, but to DNA running as a smear throughout the lane, which probably represents contamination of the λ DNA with chromosomal DNA of *E. coli.* These observations indicate that even the relatively large amounts of DNA that are present in the electrophoresed

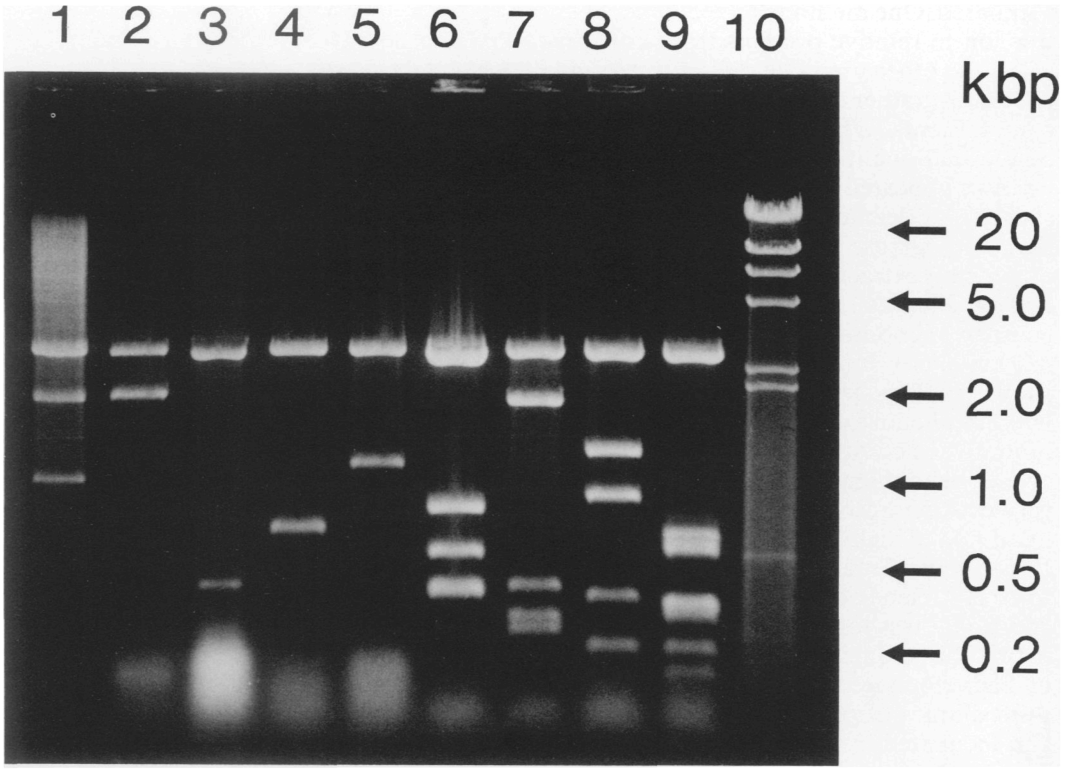

Fig. 2. 0.7% agarose gel stained with ethidium bromide of clone digests used for assessment of copy number and phylogenetic screening. Lanes (1) through (9), randomly selected plasmid library clones of *Menippe adina* digested with EcoR I and either Sal I [lanes (1) through (5)] or Hind III [lanes (6) through (9)]. Clones used were pMACC37, pMACC36, pMACC35, pMACC34, pMACC33, pMARL5, pMARL4, pMARL3, and pMARL1. Lane (10), Hind III digested λ DNA size standard. The cloning vector, pUC19, yielded a 2.7-kb vector fragment in each digest; other fragments consist of cloned sequences of *M. adina*. Amount of DNA loaded for each clone was adjusted to yield approximately 100 ng DNA/fragment for fragments in the 0.5–2-kb size range.

fragments of the digested clones should not produce high levels of nonspecific hybridization.

The intensity of the hybridization signal among the cloned DNA fragments of *M. adina* varied from undetectable to extremely dark, with several distinguishable intermediate levels. For some clones, such as pMARL1 in lane 2, there were markedly different levels of hybridization among the genomic fragments of a single clone. Although the base composition of fragments can also influence the extent of hybridization, we provisionally interpreted these results as follows: (1) faint or undetectable levels of hybridization—characteristic of sequences present as one or a few copies within the genome of *M. adina,* (2) intermediate levels of hybridization—characteristic of moderately repetitive sequences, and (3) very strong levels of hybridization—characteristic of highly repetitive sequences. By these criteria, we selected three clones to test these interpretations. Clone pMACC36 (Fig. 3, lane 9) was selected as a putative single copy sequence, clone pMARL4 (lane 4) was selected as a putative moderately repetitive sequence, and clone pMACC37 (lane 10) was selected as putative highly repetitive sequence.

The second method of assessing the copy number of specific cloned sequences within the genome of *M. adina* was conventional genomic Southern hybridization analysis. Restriction endonuclease digested genomic DNA of *Menippe* was electrophoresed, blotted onto nylon membranes, and probed with cloned sequences. The rationale for this approach is as follows. A restriction endonuclease that recognizes sites that do not occur within a repetitive sequence element will recognize sites that occur at various distances from the repetitive element depending on the particular sequence that sur-

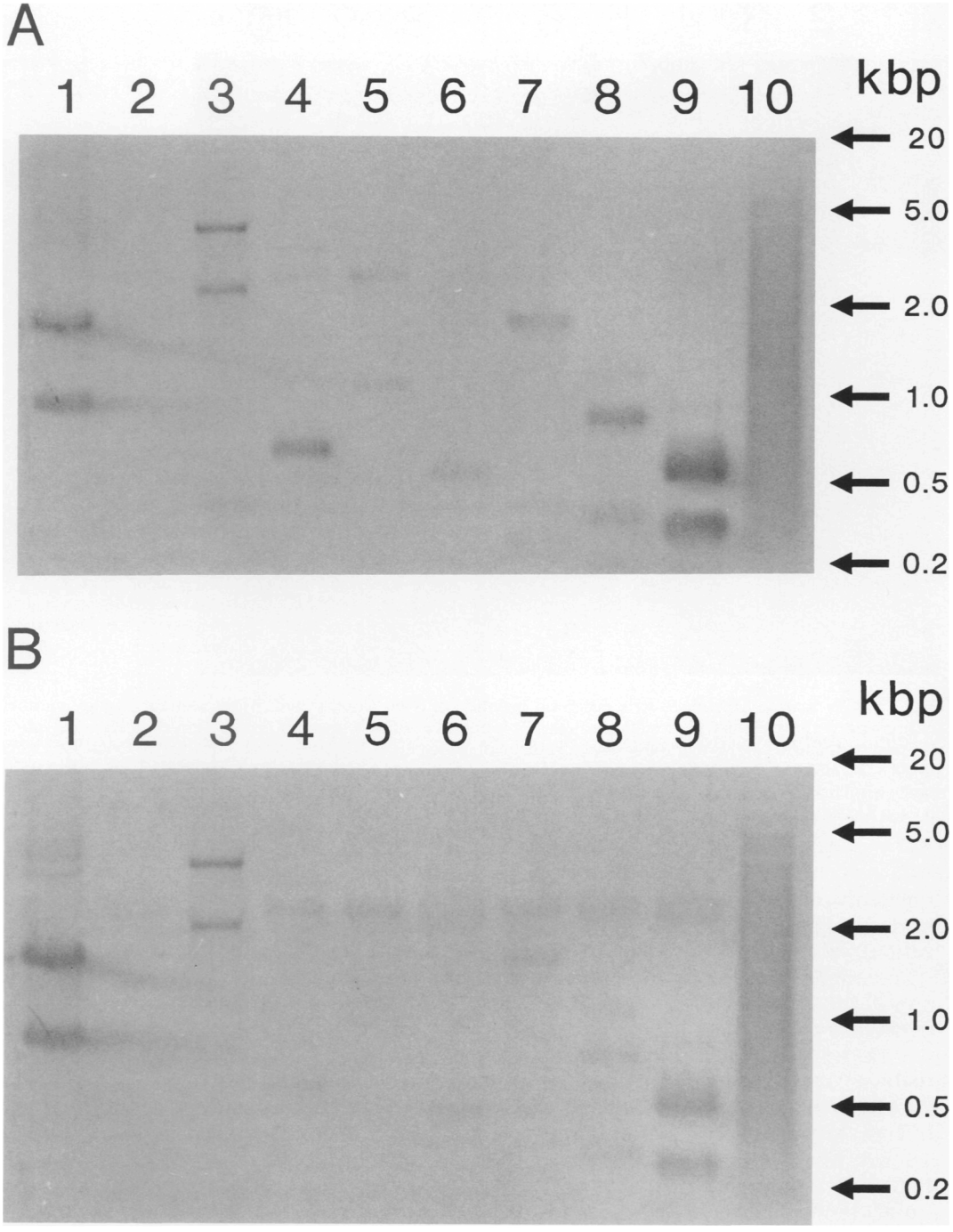

Fig. 3. Southern blot of gel in Fig. 2, hybridized with genomic DNA of *Menippe adina* (panel A), stripped and reprobed with genomic DNA of *M. mercenaria* (panel B).

rounds each occurrence of the repetitive element. A restriction fragment of distinct size would then be produced for each occurrence of the sequence within the genome, and the number of bands that appear in a Southern analysis would reflect the number of times that sequence occurred within the genome. If by chance, however, a restriction endonuclease recognized sites located within the repeated sequence, each occurrence

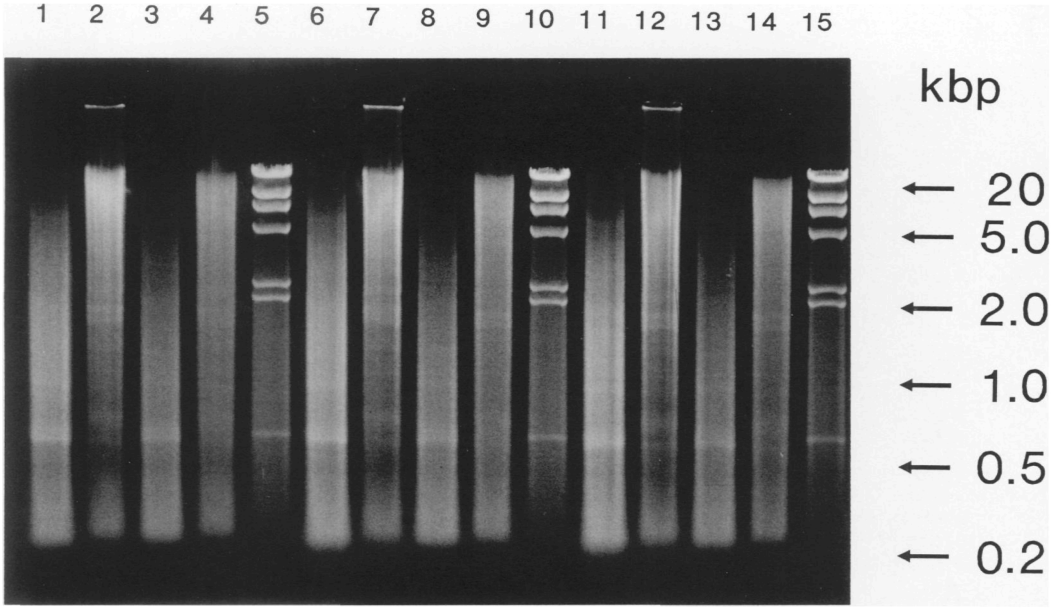

Fig. 4. Agarose gel of genomic DNA digests of *Menippe* for Southern analysis. Lanes 5, 10, and 15, size marker of bacteriophage λ DNA digested with Hind III. The remaining lanes were loaded with digests of DNA of either *M. adina* or *M. mercenaria.* Lane (1), Hae III/*M. adina;* lane (2) EcoR I/*M. adina;* lane (3) HaeIII/*M. mercenaria* and lane (4) EcoR I/*M. mercenaria.* Each digest was divided into three aliquots, and loaded into separate wells, so that lanes (1)–(4) are equivalent to lanes (6)–(9) and lanes (11)–(14). The gel was stained with ethidium bromide, photographed, and blotted onto a nylon membrane.

of the sequence would produce fragments of the same size, and the number of copies of bands appearing in a Southern analysis would underestimate the number of copies of the sequence. To guard against this possibility, we used two different restriction endonucleases for our estimates of copy number.

Figure 4 shows the agarose gel used for the Southern analysis. Four combinations of restriction endonuclease and species of *Menippe* were used, and each digest was divided into three aliquots. The faint bands visible in the genomic digest lanes indicate the presence of repetitive DNA sequences. The gel was blotted onto a nylon membrane, and the membrane cut into three pieces. Each piece, with a replicate set of lanes, was probed with a different clone. Digestions of DNA of *M. adina* and *M. mercenaria* produced similar patterns of hybridization for all three blots.

The membrane shown in Fig. 5A was probed with pMACC36, which has a genomic DNA insert of *Menippe adina* of 1.9 kb tentatively identified as a single copy sequence. For EcoR I digests of genomic DNAs of both *M. adina* and *M. mercenaria,* strong hybridization was confined to a single 2.5 kb fragment. This result is consistent with our interpretation of this clone as a single copy sequence. Because no EcoR I sites were found in the cloned portion of pMACC36 of *Menippe,* it was expected that a single genomic EcoR I fragment would contain the entire cloned region. Faint hybridization signals elsewhere in the EcoR I lanes may have resulted from hybridization with fragments of fortuitous sequence similarity, or nonspecific hybridization to repetitive DNA fragments present at high concentrations in the gel. pMACC36 strongly hybridized to two Hae III fragments of approximately 0.9 kb and 1.0 kb in size. This result is also consistent with our interpretation of a single copy sequence. The Hae III pattern would be produced by hybridization to a single region of 1.9 kb, spanned by the two Hae III fragments. Faint nonspecific hybridization signals that appear in the Hae III lanes could represent either nonspecific hybridization or specific hybridization to fragments that overlap only a small portion of the cloned probe sequence.

Figure 5B shows the membrane probed

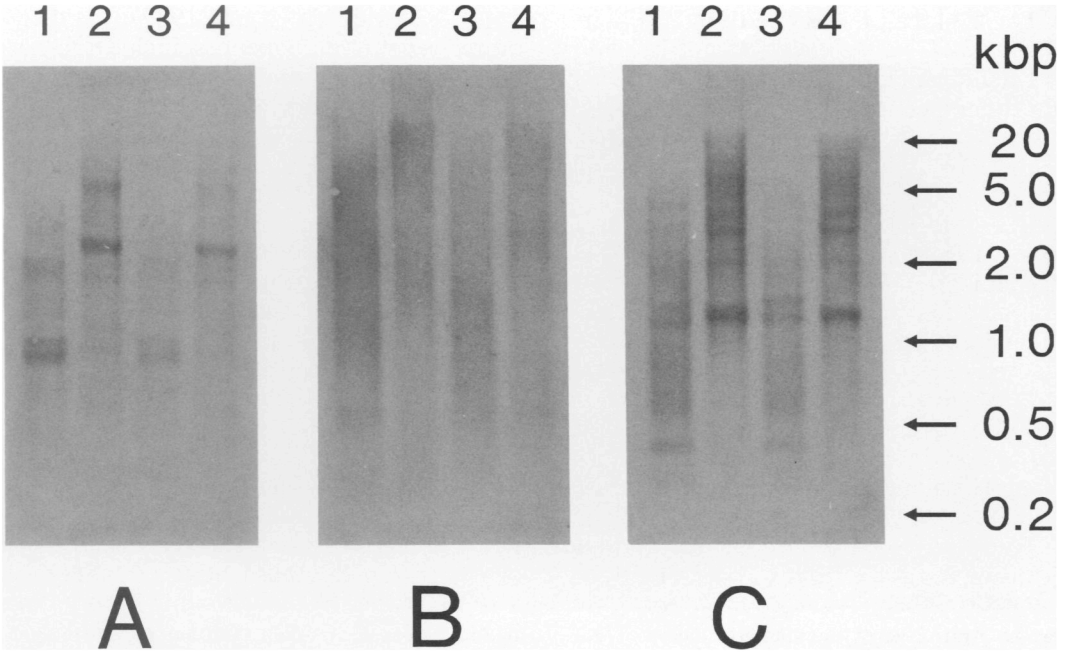

Fig. 5. Southern blots of genomic DNAs of *M.adina* and *M. mercenaria* probed with DNA clones of *Menippe adina*. Probes used were pMACC36 (panel A), pMACC37 (panel B), and pMARL4 (panel C).

with pMACC37, a putative highly repetitive sequence. No bands appear in any of the lanes, although a relatively dark smear of hybridization extends throughout each lane. This is the pattern expected for a short highly repetitive sequence that is dispersed throughout the genome. An additional observation that is consistent with pMACC37 containing a highly repetitive sequence is that this plasmid was observed to have an unusually low density during purification by cesium gradient centrifugation. This resulted in some contamination with chromosomal DNA of *E. coli* (noticeable as a smear in Fig. 2, lane 1). Short, highly repetitive satellite sequences often have anomalous densities as a result of unusual base compositions.

The membrane shown in Fig. 5C was probed with pMARL4, which was initially characterized as containing a moderately repetitive sequence of *M. adina* within a fragment of approximately 1.7 kb, along with several fragments of single copy DNA. Several EcoR I fragments produced strong hybridization signals. The 1.3 kb fragment that showed the most intense signal may represent hybridization from a single copy region within the pMARL4 sequence. In addition to the bands, a smear of hybridization in the region of the lane corresponding to fragments from 2.5 kb to at least 20 kb is also evident. This may indicate that a large number of different fragments share some similarity to the cloned sequence. In the Hae III digest lanes, about 7 bands of different intensities can be identified, along with a smear of hybridization in the region of the lane corresponding to fragments in the range between 0.5 and 3.6 kb. This pattern of hybridization would be expected if pMARL4 contained both a sequence present in one or a few copies, which produced the major bands of hybridization, along with a highly repetitive sequence element that produced a smear similar to that seen in Fig. 5B.

Phylogenetic Screening

The Southern blot shown in Fig. 3A, which was probed with genomic DNA of *Menippe adina,* was later stripped of this probe and reprobed with genomic DNA of *M. mercenaria* (Fig. 3B). A comparison of these two blots revealed differences in levels of hybridization for some of the cloned fragments. For example, in lane 7, relatively strong hybridization of genomic DNA of *M. adina* to a 0.7-kb fragment of clone

pMACC34, can be seen, although genomic DNA of *M. mercenaria* did not hybridize at a significant level to this fragment. A similar difference is also seen in lane 6.

Discussion

Genomic DNA of high purity could be isolated from stone crab testes without deproteinization by phenol extraction. Elimination of this step from our purification procedure provided several advantages. Increased yields of high molecular weight DNA were obtained, the time required to process samples was considerably shortened, and the expense and inconvenience of working with phenol (which is toxic and volatile) was eliminated.

This study was also facilitated by the development of commercially available reagents for labeling nucleic acid probes with the hapten, digoxigenin, and reagents for the detection of theses probes. Because the probes were labeled by a nonradioactive method, they could be stored indefinitely and handled without special safety precautions. In contrast with our experience with biotin-labeled probes, digoxigenin-labeled probes were extremely sensitive (less than 1 pg can be detected), could be visualized with very low background, and allowed hybridization membranes to be stripped and reprobed repeatedly.

Preparation of genomic DNA for cloning often includes a step to eliminate repetitive DNA. This is useful when a genomic DNA library is being prepared for the cloning of specific single copy sequences. By excluding repetitive DNA, the search is narrowed. However, for the purpose of obtaining random hybridization probes to be used in crustacean evolutionary studies, exclusion of repetitive sequences may be unnecessary or even undesirable. Our results with *Menippe* suggest that random single copy sequences can be easily obtained from a library of total genomic DNA. One of the nine clones that was analyzed appeared to contain only repetitive DNA (pMACC37), while most of the others contained both single copy and repetitive regions. The importance of repetitive sequences in evolution should not be overlooked. The presence or absence of specific repetitive sequence families may be useful as phylogenetic characters (Dowsett and Young, 1982; Strachan *et al.*, 1982; McLain *et al.*, 1987). Furthermore, repetitive sequences include transposable elements and retroviruses, which may impose barriers to hybridization (Bingham *et al.*, 1982), increase mutation rates (Never and Saedler, 1977), and produce other effects of potential evolutionary significance (Syvanen, 1984). The genomes of brachyuran crabs are known to contain large proportions of repetitive DNA (Vaughn, 1975; Holland and Skinner, 1977; Christie and Skinner, 1979) and at least one highly repetitive sequence appears to be conserved within Crustacea, including *Menippe* (Wang and Skinner, 1989).

Wichman and co-workers (1985) have developed the "Φ-screen" method to identify differences in the abundance of repetitive sequences among the genomes of related species. Southern blots of randomly selected clones from a genomic library that include repetitive sequences are hybridized with labeled genomic DNA from each species. Because the amount of hybridization to each cloned sequence depends on the concentration of that sequence in the genomic DNA used as a probe, repetitive sequences produce strong hybridization signals, while single copy sequences produce little or no hybridization signal. In this study we compared assessments of the genomic abundance of cloned repetitive sequences based on Φ-screen blots with results from conventional genomic Southern blots, in which the cloned sequences were used as probes. Our results suggest that the Φ-screen method can distinguish between single copy and repetitive sequences.

An advantage of the Φ-screen method that was apparent from our results is that different restriction fragments of a cloned sequence are assayed separately on a single blot. Four of the nine clones analyzed by this method yielded both restriction fragments that showed strong hybridization and fragments that showed little or no hybridization (clones pMARL1, pMARL3, pMARL4, and pMACC35), while only two clones (pMARL5 and pMACC36) contained only fragments that did not produce levels of hybridization above that for a nonspecific control (pUC19). In contrast, to obtain the same information by genomic Southern blot analysis would have required the sequential application of separate probes for each of the 22 restriction fragments seen

in Fig. 3 that represent cloned sequences of *Menippe.* Thus, Φ-screen blots can be used to identify fragments that can be subcloned and used as single copy sequence probes.

A potential limitation of the Φ-screen method may be in the assessment of the number of copies of a sequence within a genome. This became apparent in our analysis of clone pMARL4, which contains a 1.7 kb restriction fragment that produced a hybridization signal of intermediate intensity. Our initial assessment was that this fragment represented a moderately repetitive sequence, corresponding to the level of hybridization observed in the Φ-screen analysis. However, a genomic Southern analysis suggested another explanation. The pattern expected for a single copy sequence (one to several strongly hybridizing bands) was present along with the pattern expected for a highly repetitive sequence (a continuous smear). In addition, the level of hybridization within the smear was low, which would occur if only a relatively small portion of pMARL4 contains a highly repetitive sequence. Thus, the intermediate level of hybridization observed in the Φ-screen blot may reflect the total length of repetitive elements within a clone as well as the degree of repetition of those elements within the genome. As suggested by Wichman *et al.* (1985), use of more than two restriction endonucleases to fragment the cloned sequence into numerous small pieces should increase the likelihood of isolating highly repetitive sequences within separate fragments.

Cloned sequences of *Menippe adina* were probed with genomic DNA of both *M. mercenaria* and *M. adina* to screen for repetitive sequences. Differences in the intensity of hybridization to certain fragments were apparent, with the most striking seen for pMACC33 and pMACC34. The DNA inserts of these clones of *M. adina* (released as single fragments) showed no significant hybridization signal with genomic DNA of *M. mercenaria* (Fig. 3B), but intermediate levels of hybridization with genomic DNA of *M. adina* (Fig. 3A). Several possible explanations for these differences should be considered before concluding that they represent phylogenetic differences. They could represent artifacts arising from nonuniform retention of target DNA during the stripping of the membrane, or nonuniform hybridization. Another possibility is that during the cesium purification of the genomic DNA samples, a repetitive DNA sequence present as a satellite band in the cesium density gradient was inadvertently missed from the DNA sample of *M. mercenaria.* Furthermore, even if the possibilities of such artifacts could be eliminated, copy numbers of these sequences could be characteristic of the two individuals that were used in this experiment rather than the species that they represent. Demonstration of a species-specific difference in sequence copy number will thus require repetition of this experiment with other genomic DNA samples. Nevertheless, major differences in the copy number of repetitive sequence elements are not unexpected. In *Drosophila,* for example, the number of copies of the P element per genome can change extremely rapidly, and marked differences can develop in the phylogenetic distributions of these transposable elements (Bingham *et al.,* 1982). Phylogenetic differences in the occurrence of the *Mys* family of transposable elements have been demonstrated for rodents (Wichman *et al.,* 1985).

The methods described in this paper provide the necessary tools to assess several forms of nuclear sequence variation in *Menippe,* and offer techniques that should be applicable to evolutionary studies of other crustaceans as well. Single copy probes can be used in genomic Southern analysis to detect both intraspecific and interspecific variation at a virtually unlimited number of restriction endonuclease sites. A survey of the distributions of several restriction fragment length polymorphisms in populations of *M. adina, M. mercenaria,* and hybrids between these species is currently underway. Probes that detect repetitive sequence elements can reveal an otherwise unobserved component of genetic variation that may directly affect speciation and hybridization processes, as well as provide useful phylogenetic markers. Further characterization of the clones of *M. adina,* pMACC33 and pMACC34, will determine if they indeed represent repetitive sequence families that have diverged after speciation, and their relationships to other known repetitive sequence elements.

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