Development, characterisation, inheritance, and cross-species utility of American lobster (*Homarus americanus)* microsatellite and mtDNA PCR–RFLP markers

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Abstract: Effective management of exploited species demands contemporary knowledge of population structure and mating patterns. Genetic markers can prove useful in providing this knowledge. Despite its commercial importance, genetic markers for American lobster (*Homarus americanus*) are limited. We developed 12 tetra- and 1 trinucleotide microsatellite loci for American lobster that exhibit little stuttering after PCR amplification. Gene diversity of these loci ranged from 0.516 to 0.929. A four-locus multiplex permits rapid genotyping of progeny in parentage experiments with a paternity exclusion probability over the four loci of 97.8%. We examined the loci for conformity to Hardy–Weinberg expectations (HWE) and linkage using individuals from one location and found that four loci deviated from HWE. We also tested inheritance and pairwise linkage using 48 embryos from each of two females. With the exception of two loci that were derived from the same clone and separated by 72 bp, no evidence of linkage was found. We, for the first time, demonstrate the occurrence of multiple paternity in American lobster. We also observed an apparent occurrence of dispermic androgenesis, possibly the first documentation of such an event within a species. Ten of the loci amplified in European lobster (*Homarus gammarus*), although two were monomorphic and one deviated significantly from HWE. We quantified mitochondrial DNA (mtDNA) sequence variation through the use of PCR amplification of two DNA fragments, followed by digestion with restriction enzymes; eight haplotypes were detected. One of the two fragments amplified in European lobster. Both sets of markers should prove useful for population discrimination purposes, and the microsatellites, in particular the four-locus multiplex, should prove highly amenable to rapidly addressing questions about mating patterns.

Key Words: low-stutter microsatellites, mtDNA PCR–RFLP, multiple paternity, dispermic androgenesis, American lobster.

Résumé : Une gestion efficace d'espèces exploitées commercialement nécessite une connaissance de la structure des populations et de leur reproduction. Des marqueurs génétiques peuvent s'avérer utiles en vue de l'acquisition de telles connaissances. Malgré son importance sur le plan commercial, peu de marqueurs génétiques sont disponibles pour le homard américain (*Homarus americanus*). Les auteurs ont développé 12 locus marqueurs à motif tétranucléotidique et un locus trinucléotidique qui montrent peu de bandes écho suite à l'amplification PCR chez le homard américain. La

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diversité génique chez ces locus variait entre 0,516 et 0,929. Une réaction en multiplexe avec quatre marqueurs permettait de génotyper rapidement la progéniture lors d'expériences visant à identifier les parents, le tout avec une probabilité d'exclusion paternelle de 97,8 % sur l'ensemble des quatre locus. Les auteurs ont vérifié si les locus se conformaient aux attentes Hardy-Weinberg (HWE) ainsi que la liaison en utilisant des individus provenant d'un site. Quatre des locus montraient une déviation significative par rapport aux HWE. Les auteurs ont également vérifié l'hérédité et la liaison des locus deux à deux en employant 48 embryons provenant de deux femelles. À l'exception de deux locus qui avaient été isolés sur le même clone à 72 pb l'un de l'autre, aucune évidence de liaison génétique n'a été observée. Les auteurs montrent pour la première fois un cas de paternité multiple chez le homard américain. Ils ont également observé un cas apparent d'androgénèse dispermique, potentiellement la première documentation d'un tel événement au sein d'une espèce. Dix des locus ont été amplifiés chez le homard européen (*Homarus gammarus*), bien que deux d'entre eux étaient monomorphes et que l'un d'eux montrait une déviation significative par rapport aux HWE. La variation de la séquence de l'ADN mitochondrial (mtDNA) a été quantifiée en amplifiant deux régions d'ADN et en les analysant par digestion enzymatique. Huit haplotypes ont ainsi été identifiés. Une des deux régions pouvait également être amplifiée chez le homard européen. Ces deux jeux de marqueurs devraient s'avérer utiles pour différencier les populations et les microsatellites, en particulier les quatre locus employés en multiplexe, devraient permettre de répondre rapidement à des questions sur les accouplements.

Mots clés : microsatellites à faible écho, ADNmt, PCR–RFLP, paternité multiple, androgenèse dispermique, homard américain.

[Traduit par la Rédaction]

Introduction

American lobster (*Homarus americanus*, Nephropidea; clawed lobster) occur at intertidal depths to 720 m (most frequently 4–50 m) along continental shelves throughout much of the Western North Atlantic from southern Labrador to offshore North Carolina. American lobster is commercially important, having the greatest landed value of any fishery in Canada (CDN $442.5 million in 1998). Effective management of exploited species requires the identification of biologically relevant management units that reflect their degree of reproductive isolation. Genetic markers have proven to be a valuable tool for such purposes in some marine species (as reviewed by Carvalho and Hauser 1994). Because of the potential importance of breeding structure in influencing effective population size and, as a consequence, the degree to which subpopulations are genetically differentiated (e.g., Wright 1938), quantitative knowledge of mating structure is also essential. Genetic markers have also proven invaluable in elucidating mating patterns (e.g., Westneat et al. 1987; Burke 1989).

Attempts have been made to study both population and mating structure of American lobster with genetic markers. From these studies, the inability to reject the null hypothesis of no differences among populations of American lobster might suggest that little population structuring exists, but the limited resolution of the markers available for American lobster at the time (e.g., allozymes, Tracey et al. 1975; noting concerns raised by Shaklee 1983; RAPDs, Harding et al. 1997) were likely a major contributing factor. Evidence from morphometric and other studies suggest that there may be differences among populations (e.g., Harding et al. 1993), although such differences may simply reflect environmental effects. The mating pattern in American lobster is similarly unresolved. Unexpected allele frequencies at one allozyme locus led to the suggestion of multiple paternity of the embryos of 1 out of 32 females (Nelson and Hedgecock 1977), although Tam and Kornfield (1996) did not find any evidence of multiple paternity in 13 females. No attempt has been made to address more complex questions such as the factors affecting individual reproductive success. Additional variable, robust, genetic markers are required to better quantify population structure and the mating pattern of this commercially important species.

Here, we describe two classes of genetic markers useful for the study of American lobster biology: 12 tetra- and 1 tri-nucleotide repeat microsatellite loci, as well as primers designed to amplify two approximately 3-kb regions of mitochondrial DNA (mtDNA) for which we used restriction enzymes to identify variation. We screened individuals from one location to assess the utility of these microsatellite loci for population analyses and checked for Mendelian inheritance and linkage among these loci by genotyping embryos from two embryo-carrying (berried) females. We describe one four-locus multiplexing system that allows rapid genotyping for studies in mating structure. We also assess the potential utility of both classes of markers to address similar questions on the closely related European lobster (*Homarus gammarus*).

Materials and methods

Microsatellites

Microsatellites were isolated as outlined in Hamilton et al. (1999) with minor modifications as described in McPherson et al. (2001). Briefly, DNA was obtained from ethanol-preserved muscle using phenol–chloroform extraction methods, digested with *Hae*III, dephosphorylated, and ligated to the double-stranded SNX linker complex (Hamilton et al. 1999) in the presence of *Xmn*I. Ligation products were hybridized to 5′-biotinylated oligonucleotides ($(GACA)_4$ and $(GATA)_4$) at 48°C for 30 min, then complexed with streptavidin-coated magnetic beads (Dynabeads, Dynal, Oslo, Norway) that had been previously washed with PBS – 0.1% bovine serum albumin (BSA). The bead-hybridization mixture was incubated at 43°C for 1 h to allow beads to bind

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Table 1. Array sequence, primer sequence (A-labeled primer; in case of multiple useful alternate primers, preferred primer in bold), optimal annealing temperature (T_A (°C) (temperature range in which acceptable product amplification occurred)) and Genbank accession number for the *Ham* loci.

Locus	Array sequence	Primer	Sequence (5′→3′)	T_A (°C) (range)	Genbank accession No.
Ham6	$(gATA)_6gATg(gATA)_6ggTA(gATA)_4$	A	CATgCAggTATACACAgACACACTC		AF440472
		B1	TCggAATCTAACgCTTgACC	60	
		B2	ACTgTgTTgACTTAATCTggAgAAA	60 (58–60)	
Ham9	$(gATA)_3CCgTAgATAgACAggTATTgTA(gATA)_4CTgTA(gATA)_4CTgTA(gATA)_3TAgATATTTAgATAggTA(gATA)_2$	A	CTggCTCCATgCATACCC		AF440473
		B1	CCggAgATCACgTgTgAgA	58 (52–60)	
		B2	CAAgAggCTACATAACTTTTCgTg	60 (52–60)	
Ham10	$(gACA)_5AACAgACAgACg(gACA)_4$	A	CTATCTACAAggTCATATgTTCAgTT		AF440474
		B1	CTCCCATCgTCCTgCTC	52 (50–56)	
		B2	CACAACACACCTTTTATACgATT	50 (50–54)	
Ham15	$(CA)_5(TA)_2CA(gACA)_{12}$	A	CTgCgCCATTAgAggACA		AF440475
		B1	gCATggggTTCAgTgTTg	52	
		B2	gTTgCCATCAgggTgTTC	52	
Ham21	$(gACA)_4gATTA(gACA)_6$	A	TTACTCACTCAACggCACT		AF440476
		B1	TCTTCCCTCAAACATTCAC	52	
		B2	gACTTgCggTgTgAAAA	56	
Ham22	$gACACA(gACA)_4gTgACAgAAATAgAggCAgACA(gA)_3AATAAA(gACA)_4(gA)_2$	A	gAggCAAACATACAAATAgACACA		AF440477
		B1	gTTTgTCCCCTTATTTTCTggT	56 (56–58)	
		B2	ACTAgCTgAAgTATCACCTgAAAgA	58 (50–60)	
Ham30	$gA(gACA)_4gAAAgACAgAAA(gACA)_7gAgA(gATA)_3(AT)_2$	A	CCTTTTATATTCTATCTATCTATCTCTg		AF440478
		B1	gACCAgACCCgTgAgTT	56 (52–60)	
		B2	gTTTAACCggACCAgAC	56 (52–60)	
Ham32	$(TAgA)_2CAgATAgATTgAAAgAATAACC(gACA)_3gCCAgATC(gATA)_2gATTgAAA(gA)_2AACA(gACA)_2(gA)_2AA(gACA)_2gACTgATAgAAA$	A	CAggggTgAAggTAgATAgA		AF440479
		B2	CTCggAAgATTAATgAACAAA	50 (50–56)	
Ham42	$gA(gACA)_4AgCA(gACA)_4AA(CA)_4AACA(gACA)_3(CA)_3$	A	ggAATCACTgTCTggCTgT		AF440480
		B1	gTTCAgTATAACgTCACATCACTC	52	
		B2	gTATAACgTCACATCACTCTAATg	52	
*Ham44**	**$CA(gACA)_4AACg(gACA)_3CATAACAgCCAAgTCTgC$**$AgCgAAATTAACA(ACAg)_3CCCAACAgCCAAgTTTACAgTgATATAACCAACAgAATAACAgACAAA(gACATACA)_3$	A	TTCTCCTgTggTgATACgAA		AF440481
		B2	TAggTATgggCgggAgT	52 (52–54)	
		B3	gCAgACTTggCTgTTATgTg	58 (52–58)	
Ham48	$CA(gACA)_3gA(gACA)_7gA(gACA)_3$	A	TTCTgAAAgTTTgACgggTTA		AF440482
		B1	ACACgTACACACAgggATTg	54	

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Table 1 (*concluded*).

Locus	Array sequence	Primer	Sequence (5′→3′)	T_A (°C) (range)	Genbank accession No.
Ham53	gTTgCT(gTT)$_5$gTC(gTT)$_8$	A	ggCATCCCATagTgAAgg		AF440483
		B1	ATTTgCgTTTTTgTTTCATTT	58 (56–58)	
Ham54	CA(gACA)$_2$(gA)$_2$(gACA)$_8$	A	AR†gATAATTCATAgCAgACAgACAgA		AF440483
		B4	ACAAAAACgCAAATTAAAAAgTCA	55	

*Sequence in bold amplified using B3.
†R = A and g (both bases detected in different sequences of this locus).

to DNA–oligo hybrids then washed three times (using a new tube for each wash) with decreasing concentrations of standard saline citrate (SSC; 2×, 1×, and 0.5× with 0.5 ng of forward linker primer/μL) at 48°C. After washing, enriched DNA was released after incubation with 30 μL low TE (10 mM TrisHCl, 0.1 mM EDTA (pH 8.0)) at 95°C for 15 min. The PCR profile (1 × 92°C for 5 min, 40 × (94°C for 45 s, 62°C for 1 min, and 72°C for 2 min), and 1 × 72 °C for 30 min) included a 30-min extension step that generated a poly(A) overhang. PCR products were purified using Qiaquick PCR columns (Qiagen, Valencia, Calif.) and ligated into TOPO 2.1 TA vector (Invitrogen, Carlsbad, Calif.). Invitrogen ONESHOT *Escherichia coli* were transformed with recombinant plasmids and plated at low density on warm Luria–Burtani plates containing 50 μg ampicillin/mL. Colonies were size selected for inserts of greater than 400 bp by growing overnight and screening via PCR and electrophoresis on 1.5% w/v agarose gels.

Size-selected plasmid DNA was prepared using a Qiaprep spin miniprep kit (Qiagen). Cycle-sequencing reactions using a universal reverse primer, Thermo-SequenaseII (Amersham Pharmacia Biotech, Piscataway, N.J.), and the following conditions: 25 cycles of 96°C for 30 s, 50°C for 15 s, and 60°C for 1 min were performed on a Techne Genius thermocycler (Techne Inc., Princeton, N.J.). Sequence was detected on an ABI 373 A DNA sequencer (Applied Biosytems, Foster City, Calif.). Primers flanking microsatellite regions were designed with the assistance of Primer3 (Rozen and Skaletsky 1998). For one flanking region, a single primer was ordered 5′ labeled in HEX while multiple primers were designed for the other flanking region. For most loci, the selection of which flank to assign the labeled primer was based on the limited availability of sequence for that one flank, consequently limiting the options for primers. When similar quantity of sequence was available for both flanking regions, the labelled primer was assigned to the flank that had a single, apparently more stable primer.

Microsatellites were amplified using the following conditions: 5× (92°C for 1 min, annealing temperature + (5°C, 4°C,…, 1°C) for 30 s, 72°C for 15 s); 25× (92°C for 1 min, annealing (Table 1) for 30 s, 72°C for 15 s); and 72°C for 30 min on a PTC-100 MJ Research thermocycler (MJ Research, Waltham, Mass.). Reactions were carried out in 10-mL volumes in 50 mM KCl, 20 mM Tris (pH 8.4), 0.2 mM each dNTP, 0.5 mM of each primer (5′ end labeled with HEX), 20–100 ng template DNA, and 0.8 U *Taq* DNA polymerase. Amplified products and a 4-bp allelic ladder were electrophoresed at 850 V on 0.8 mm, 8% w/v denaturing-polyacrylamide gels for approximately 1–2 h. DNA fragments were visualized on an FMBIO II® fluorescent imaging system (Hitachi Software Engineering America Ltd., San Francisco, Calif.).

Observed heterozygosity, gene diversity, mean number of alleles, conformity to Hardy–Weinberg expectations (HWE), and pairwise linkage disequilibrium were analysed in 50 American lobster from the Crowell Basin, Gulf of Maine, and 47 European lobster from Norway using GENEPOP version 3.2a (Raymond and Rousset 1995). Conformity to HWE was tested using the exact test of Guo and Thompson (1992). When the number of alleles at a locus in a collection was less than five, the complete enumeration (Louis and Dempster 1987) was used. For loci with more than four alleles in a collection, a Markov chain method was used to estimate the exact *P* value (Guo and Thompson 1992). Paternity exclusion probabilities were calculated for each locus and cumulatively for several loci following Weir (1996). Mendelian inheritance and direct tests for linkage disequilibrium were assessed by χ^2 analyses using genotypes of two berried females, those of their embryos, and the inferred paternal genotypes.

mtDNA

To identify appropriately sized target fragments in the American lobster mtDNA genome, published American lobster mtDNA sequences (Boore et al. 1995; GenBank accession Nos. U29717, U29718, and U29719) were aligned to the homologous regions of the mtDNA genome of giant shrimp (*Penaeus monodon*), the most closely related species with its entire mtDNA genome sequenced (Wilson et al. 2000; GenBank accession No. NC002184). Primers were designed from the American lobster sequences to attempt to amplify adjacent fragments each expected to be approximately 3 kb in length. Primers were again designed with the assistance of Primer3 (Rozen & Skaletsky 1998). Multiple primers pairs were designed for each fragment.

Fragments were amplified using the following conditions: 94°C for 3 min; 39 × (94°C for 30 s, annealing temperature for 30 s, 72°C for 210 s); and 72°C for 5 min on a PTC-100 MJ Research thermocycler. Optimal annealing temperatures and primer combinations were determined by conducting the amplifications on four individuals at six temperatures (51–61°C at 2°C increments). Reactions were carried out in 25-μL volumes of 50 mM KCl, 20 mM Tris (pH 8.4),

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0.2 mM of each dNTP, 0.5 mM of each primer, 20–100 ng template DNA, and 1.25 U *Taq* DNA polymerase.

The PCR products were digested with eight restriction enzymes, *Dde*I, *Dra*I, *Eco*241, *Hin*dIII, *Hin*fI, *Mbo*I, *Rsa*I, and *Taq*I, according to the manufacturer's instructions. The resulting fragments were separated on 2% agarose gels for 5–6 h at 90 V, stained with ethidium bromide, and imaged under UV light with a Polaroid camera. The mtDNA fragment sizes were in reference to a 100-bp DNA ladder run in the same gel.

Results

Of 336 screened colonies, 254 (134 of 161 and 120 of 175 of the GACA and GATA libraries, respectively) had inserts greater than 400 bp. Of these colonies, 70 were sequenced (45 and 25, resepectively, from the two libraries), 47 of which (42 and 5, respectively) contained microsatellites, although 14 (13 and 1) had insufficient flank with which to design primers, and a further 11 (9 and 2) were duplicated sequences. Primers were designed for 22 potential loci, of which 9 proved monomorphic. Of the 13 variable loci (Tables 1 and 2), 1 (*Ham42*) appeared to exhibit null alleles at a high frequency (H_e = 0.842, H_o = 0.250) and was not examined beyond the initial temperature trials. Another locus (*Ham44*) exhibited large alleles along with, at times, difficult to interpret artefact bands. Consequently, an additional unlabeled primer was designed to amplify only a portion of the sequence of this locus (see Table 1). All of the loci produced clearly distinguishable alleles with limited stutter. The majority of the repeat units in these arrays were tetranucleotides; however, many of the loci had alleles that differed in size by less than the 4 (or 3) bp (Table 2; Fig. 1). The alleles not following the standard 4-bp size difference at *Ham10* and *Ham21* were found to occur within 1 bp of standard alleles (Fig. 1). Alleles at *Ham6* appeared to fall into two categories: (*i*) alleles that differ from most other alleles by multiples of 4 bp exhibiting a faint stutter band 4 bp below the primary band and (*ii*) alleles of intermediate size that generally exhibit two equally intense bands 2 bp apart (Fig. 1). Gene diversity of the 13 loci ranged from 0.516–0.929 and the number of alleles varied from 5–24 per locus (Table 2). Three loci appeared to deviate from HWE (*Ham21*, $P < 0.0001$; *Ham22*, $P < 0.0001$; *Ham44B2*, $P = 0.0110$ (not significant after correcting for multiple testing (Rice 1989)); *Ham44B3*, $P = 0.0039$), and there was weak evidence of linkage disequilibrium between two pairs of loci (*Ham21* and *Ham32*, $P = 0.0488$; *Ham30* and *Ham32*, $P = 0.0358$; neither test was significant after correcting for multiple tests (Rice 1989)).

Ten of 12 primer sets tested in European lobster (*Homarus gammarus*) produced a detectable product (Table 2); neither *Ham6* nor *Ham22* would amplify. Two loci (*Ham10* and *Ham15*) were monomorphic. One locus (*Ham30*) appeared to have only partial amplification success because many samples failed (Table 2) and this locus deviated from HWE ($P < 0.0001$). *Ham53* and *Ham54* were in significant linkage disequilibrium ($P < 0.0001$) and there was weak evidence of linkage disequilibrium between *Ham9* and *Ham21* ($P = 0.0463$; not significant after correcting for multiple tests (Rice 1989)).

Table 2. Sample size, allelic size range (in base pairs), number of alleles detected, minimum size difference between alleles, gene diversity (H_e), observed heterozygosity (H_o), and paternity exclusion probability (PE) for American and European lobster at 14 microsatellite loci isolated from American lobster.

	American lobster						European lobster					
Locus	Sample size	Allele size range (bp)	No. of alleles	Min. bp diff.	H_e	H_o	PE	Sample size	Allele size range (bp)	No. of alleles	Min. bp diff.	H_e	H_o	PE
Ham6	48	106–164	21	2	0.929	1	0.837	47	n.a.	0	n.a.	n.a.	n.a.	n.a.
Ham9	47	140–202	5	2	0.516	0.574	0.197	45	162–184	4	2	0.282	0.267	0.101
Ham10	50	104–136	12	1	0.771	0.82	0.529	46	83	1	n.a.	0	0	0
Ham15	50	88–158	17	2	0.878	0.9	0.732	47	86	1	n.a.	0	0	0
Ham21	48	120–174	14	1	0.798	0.563	0.576	46	104–108	2	4	0.022	0.022	0.02
Ham22	50	154–182	5	4	0.773	0.32	0.662	47	n.a.	0	n.a.	n.a.	n.a.	n.a.
Ham30	50	76–128	7	4	0.682	0.58	0.383	37	72–132	9	2	0.514	0.297	0.193
Ham32	45	178–198	5	1	0.687	0.622	0.394	47	180–197	4	4	0.103	0.106	0.067
Ham42	8	134–218	7	2	0.842	0.25	n.a.	0	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
*Ham44*B2	49	194–258	24	1	0.934	0.796	0.845	0	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
*Ham44*B3	49	64–110	11	2	0.773	0.551	0.542	47	94–102	3	4	0.103	0.106	0.067
Ham48	50	90–166	13	2	0.787	0.8	0.559	47	96–108	4	4	0.747	0.787	0.477
Ham53	50	126–165	13	3	0.912	0.94	0.8	46	126–135	3	3	0.553	0.543	0.227
Ham54	50	70–146	14	4	0.885	0.78	0.751	47	70–118	6	4	0.642	0.702	0.327

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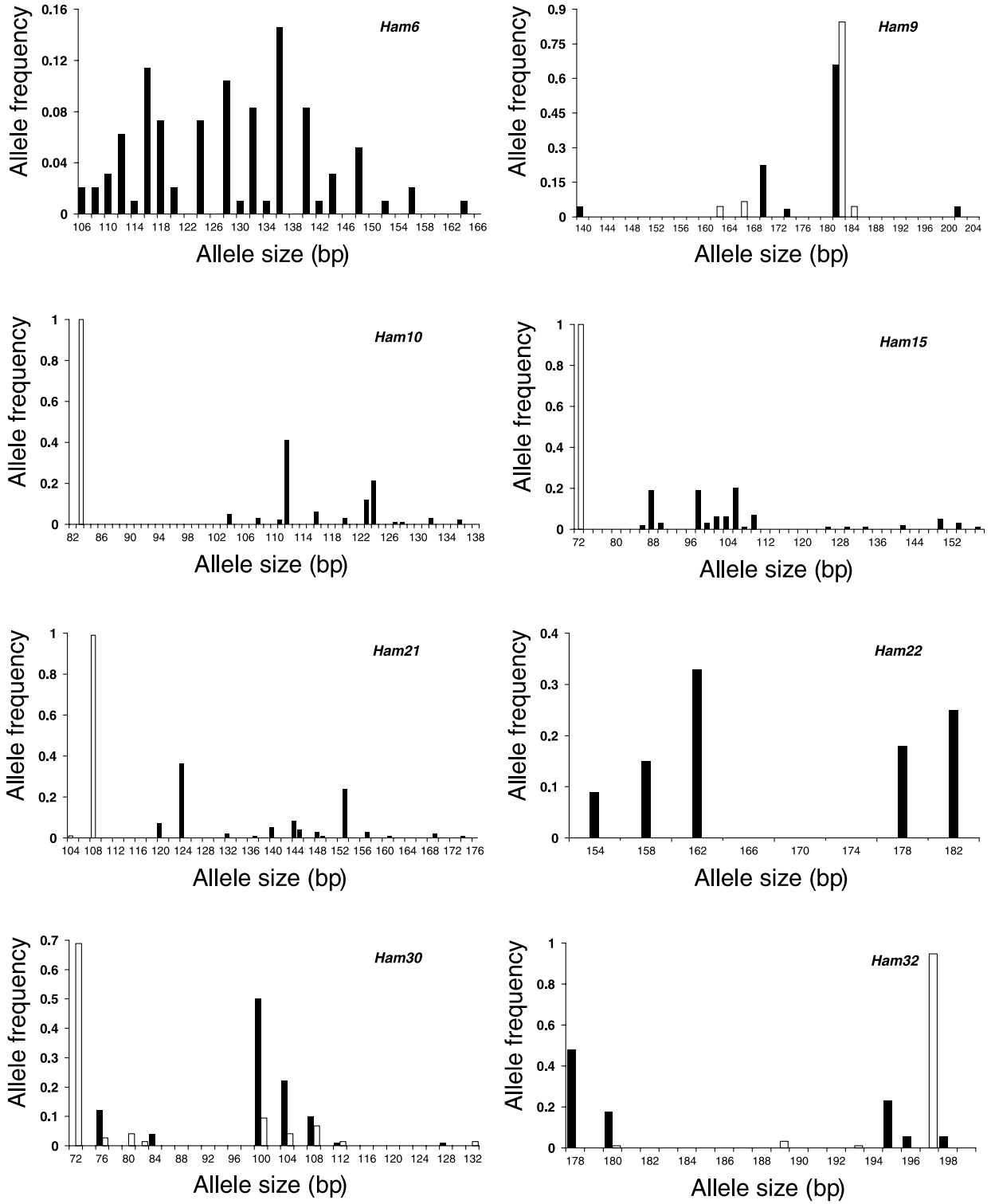
Fig. 1. Allele frequencies for American (black bars) and European (white bars) lobster.



Forty-eight embryos from a berried female originating from Grand Manan were analysed to assess Mendelian inheritance of these loci. Forty-four of the 48 embryos were consistent with two parents, whereas three embryos had genotypes consistent with fertilization from a second male. The genotypes of two of these three embryos were inconsistent with fertilization from the putative father of the other embryos at 8 of the 11 loci examined (three of four loci of the four-locus multiplex — see below) and the third at 7 of the 11 loci (two of the multiplex loci). One embryo lacked maternal alleles at all 10 discriminatory loci examined (both the mother and putative father were 178/178 homozygotes at *Ham32*), but was homozygotic for one of the common putative paternal alleles at five loci and possessed both of the

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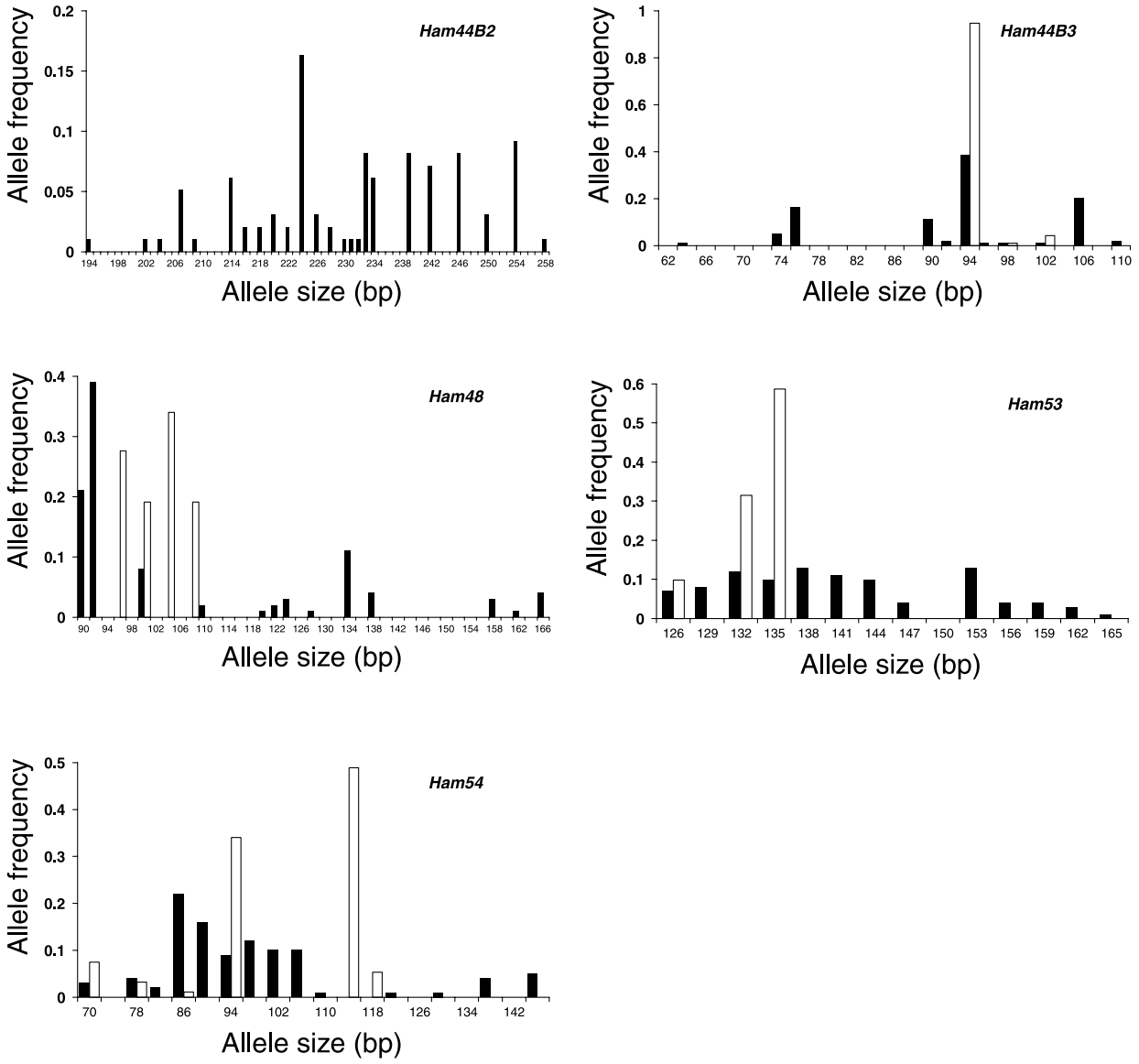
Fig. 1. (*concluded*).



putative paternal alleles at the other five. The possibility of it being an egg from another spawning can be discounted because this would necessitate the putative parents having nearly identical genotypes to the identified putative father (probability less than 1.0×10^{-10}). All four of these embryos were omitted from subsequent analyses. Assortment of alleles did not deviate from Mendelian expectations at any locus (Table 3) and, with the exception of *Ham53* and *Ham54*, which were isolated from the same clone sequence and separated by only 73 bp, there was no evidence of linkage among any loci (Table 4). *Ham53* and *Ham54* were highly linked with no recombination events detected (i.e., only four of a possible 16 genotypic classes observed in either set of embryos).

Multiplexing of loci

Most attempts at multiplexing loci in common reactions were unsatisfactory: even when loci both amplified, background intensity increased and artefact bands not present in any single locus reactions at times became prominent. However, one suite of four loci provided a rapid means of obtaining data for parentage analyses (*Ham 9*, *Ham10*, *Ham21*, and *Ham44* using an annealing temperature of 52°C). These loci were multiplexed in a single colour channel with only minimal overlap of alleles between loci using the unlabeled primers *Ham9B2*, *10B2*, *21B1*, and *44B2* with a total allelic range of approximately 108–300 bp. We also labeled *Ham9* and *Ham21* with a different fluorescent tag and used alternate unlabeled primers at *Ham9* (B1), *Ham10* (B2), and *Ham21* (B2). This resulted in a decrease of the total allelic size range (135–252) with no overlap of allele sizes among loci and further reduced multiplex generated background. In all cases, *Ham9* primer concentrations were 33% those of the other loci.

mtDNA

The PCR amplification from American lobster mtDNA generated two fragments of approximately the expected size. Although one primer pair was chosen for each mtDNA frag-

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Table 3. Chi-square goodness-of-fit tests for single-locus segregation of microsatellite DNA genotypes at 11 loci in embryos of 2 female American lobster.

Locus	Female	Maternal genotypes	Paternal genotypes*	No. of progeny	Observed ratio	Expected ratio	χ^2
Ham6	PEI	118/110	152/116	48	12:13:14:9	1:1:1:1	1.67
	GM	116/110	144/128	39	9:12:13:5	1:1:1:1	3.97
Ham9	PEI	182/170	182/182	48	1:1	1:1	0
	GM	182/182	182/170	44	5:6	1:1	0.36
Ham10	PEI	124/112	125/112	47	11:12:13:11	1:1:1:1	0.23
	GM	134/116	124/112	44	7:5:4:6	1:1:1:1	1.82
Ham15	PEI	104/88	110/104	48	7:6:5:6	1:1:1:1	0.67
	GM	154/98	98/88	43	11:14:10:8	1:1:1:1	1.74
Ham21	PEI	122/122	156/152	48	21:27	1:1	0.75
	GM	122/122	148/118	44	5:6	1:1	0.36
Ham30	PEI	108/108	104/100	48	1:1	1:1	0
	GM	108/100	100/76	44	10:7:10:16	1:1:1:1	3.98
Ham32	PEI	196/195	195/176	45	7:14:15:9	1:1:1:1	3.98
	GM	178/178	178/178	44	1	1	0
Ham44	PEI	92/74	92/74	48	13:25:10	1:2:1	0.46
	GM	92/92	104/104	44	1	1	0
Ham48	PEI	100/92	92/92	48	13:11	1:1	0.33
	GM	162/92	124/90	43	7:9:18:9	1:1:1:1	6.77
Ham53	PEI	156/132	153/138	48	13:15:13:7	1:1:1:1	3
	GM	153/129	135/132	44	6:10:17:11	1:1:1:1	5.64
Ham54	PEI	84/80	84/76	48	13:15:13:7	1:1:1:1	3
	GM	88/84	88/80	44	6:10:17:11	1:1:1:1	5.64

Note: PEI, Prince Edward Island; GM, Grand Manan.
*Paternal genotype inferred.

ment (Table 5), others also produced usable fragments (Appendix 1). Three restriction enzymes produced RFLPs (Table 6) in the first mtDNA fragment, resulting in a total of eight composite haplotypes (Fig. 2). Only the second fragment amplified in European lobster.

Discussion

The results above indicate that the genetic markers developed for American lobster will be useful for a wide range of applications for both American and European lobster. Our finding that American lobster has much higher levels of genetic variation than European lobster is consistent with the results of Tam and Kornfield (1996), providing further evidence that American lobster has had a much greater long-term effective population size than European lobster. The clearly distinguishable alleles at all of the microsatellite loci characterised infers that they can be sized with minimal error in population analyses and highlights the benefit of our selecting microsatellite loci with longer repeat unit lengths. Loci composed of dinucleotide repeat motifs, while more frequent in the genome than loci with longer repeat motifs (e.g., Schug et al. 1998), are more prone to PCR-induced stutter (e.g., Edwards et al. 1991). This can make the differentiation between homozygotes from heterozygotes with alleles differing by one repeat length difficult.

The detection of alleles that differ by 1 or 2 bp underscores the importance of having a precise means of assessing allele size and the need for sample redundancy to ensure consistent allele designation. Although multiplexing can produce data more rapidly, it should be used cautiously for analyses of individuals of unknown potential genotypes (e.g., population analyses) and only after thorough optimization and genotype verification. Amplifying more than one locus in a PCR invariably leads to greater background and often creates artefact bands (e.g., Donini et al. 1998). An al-

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Table 4. Pooled χ^2 values (Grand Manan above the diagonal, P.E.I. below) for pairwise tests of linkage between American lobster microsatellite loci.

	Ham6	*Ham9*	*Ham10*	*Ham15*	*Ham 21*	*Ham30*	*Ham32*	*Ham44*	*Ham48*	*Ham53*	*Ham54*
Ham6		6.4	16.8	17.21	3.6	18.03	n/a	n/a	17.1	16.8	16.8
Ham9	3.67		5.45	4.07	1.09	6.67	n/a	n/a	9.28	7.64	7.64
Ham10	7.81	1.17		12.44	12.73	13.93	n/a	n/a	22.12	15.64	15.64
Ham15	7.33	2.67	15.3		3.7	22	n/a	n/a	22.76	21.37	21.37
Ham21	7.67	1.09	3.74	3		12.69	n/a	n/a	9.28	10.18	10.18
Ham30	2.33	0.33	5.6	5.33	0.83		n/a	n/a	14.38	19.14	19.14
Ham32	15.8	5.67	8.69	7.33	8.16	8.51		n/a	n/a	n/a	n/a
Ham44	6.83	9.58	5.87	5.17	1.25	4.92	21.67		n/a	n/a	n/a
Ham48	1.67	0.67	2.53	2.67	1.83	0.67	10.64	4.92		22.86	22.86
Ham53	10.67	4.67	12.57	12	6.67	8.67	14.38	16.83	6.67		139.68*
Ham54	10.67	4.67	12.57	12	6.67	8.67	14.38	16.83	6.67	156*	

*$P < < 0.001$

Table 5. Primer sequence and annealing temperature for primers used to PCR amplify lobster mtDNA fragments.

Fragment	Primer	Sequence	T_A (°C)
1	HamMT0kbL3	TTgACCCTTATTTCAggAACAAT	57
	HamMT3kbR1	gTggTCAgCTgggggATAAg	
2	HamMT3kbL1	gCgTCACCCCTTATAgAgCA	57
	HamMT6kbR1	CAACCgCTTAAAATgAAgTTAgAAg	

ternative for population analyses that still achieves high throughput without necessitating as intensive an optimization process or compromising data quality is to amplify each locus separately and then combining 2–3 loci before electrophoresis.

The linked loci (*Ham53* and *Ham54*) have great potential for identifying genetic admixtures (e.g., Estoup et al. 1999). Insignificant linkage disequilibrium in the Crowell Basin samples may reflect the mixing of several subpopulations or a sufficiently large effective population size being maintained long enough for multiple lineages to develop through the accumulation of mutations at both loci. The possibility of lab error being a contributing factor can be rejected as the genotypes were verified with an initial 12% redundancy in these samples (i.e., 6 of the 50 American samples were randomly duplicated), as well as by regenotyping 32% of the samples at both loci; identical genotypes were obtained in all cases. This finding highlights the importance of conducting linkage and segregation analyses with novel loci; had we no a priori knowledge that these loci were physically linked, the use of population data alone would have resulted in the erroneous treatment of these as independent loci. The significant linkage disequilibrium detected in European lobster is consistent with a lower effective population size in that species.

For the first time, we have demonstrated the occurrence of multiple paternity in American lobster. Previous genotyping of embryos from berried females either only inferred multiple paternity based on unexpected allele frequencies at one allozyme locus (Nelson and Hedgecock 1977) or found no evidence of multiple paternity (Tam and Kornfield 1996). The detection of multiple paternity in the progeny of one of the two females ($n = 48$ for each female) used to test the inheritance demonstrates the utility of these loci to address questions about mating patterns. The four-locus multiplex developed provided the ability to rapidly assess parentage; the occurrence of additional male fertilization was detected at three of the four loci in two embryos and two of the four in the third embryo. The combined paternity exclusion probability of 97.8% can be increased to 99.6% with the addition of only one more locus (*Ham6* or *Ham53*). These markers should thus prove highly amenable to rapidly address questions relating to mating structure and individual reproductive success.

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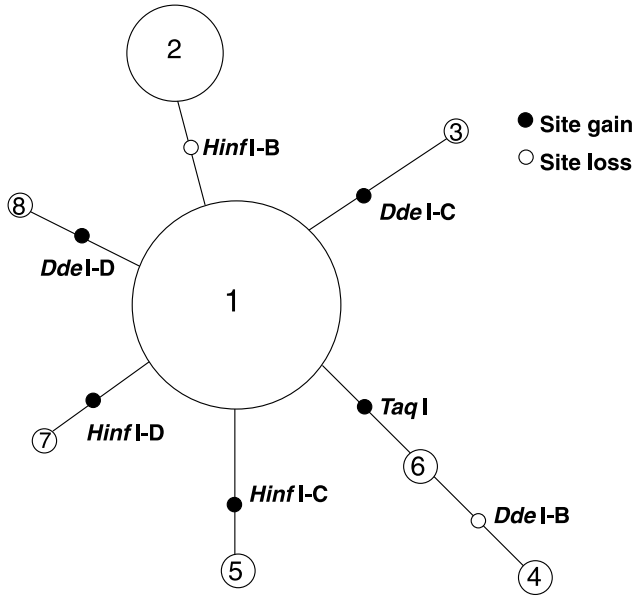
Table 6. Fragment sizes (bp) generated by the restriction enzymes used in the first fragment of American lobster mtDNA.

Restriction endonuclease	Fragment pattern	Size (kb)													
*Dde*I	A		870	605		410			290		215	120	100*		
	B	930		605		410			290		215	120			
	C		870	605		410		390			215	120			40*
	D			605	470	410	400		290		215	120	100*		
*Dra*I	A	1000	875	695	120	105									
*Hin*dIII	A	2550	170	110											
*Hin*fI	A	795		535		415	395			220	195				
	B	795	635	535			395				195			155	145
	C			535	450	415	395	345		220	195			155	145
	D	795		535			395		225	220	195		190	155	145
*Mbo*I	A	730†		480	420	180	160								
*Rsa*I	A	1300	770	520	205	100									
*Taq*I	A	930	590	490		425	250								
	B	930		490†		425	250	100*							

*Inferred fragments.
†Probable double fragment.

Fig. 2. Parsimony network of site gains and losses among mtDNA haplotypes of American lobster; circle sizes are proportional to haplotype frequency. Letter designations for each site gain and loss with the various restriction enzymes used are in reference to haplotype 1; the restriction fragment sizes for the various enzymes are given in Table 5.



The finding of one embryo lacking maternal alleles at all discriminatory loci, but possessing one or both putative paternal alleles at all loci was clearly unexpected; all other embryo genotypes consisted of a maternal and putative paternal allele at each locus. Explanations for this observation remain somewhat speculative, although it appears to be an instance of naturally occurring dispermic androgenesis, possibly representing the first such event documented within a species. Researchers routinely assessing parentage in this and other species should be cognisant of such potentially unexpected results.

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Appendix

Table A1. Alternate primer pairs for American lobster mtDNA fragments.

Fragment	Forward primer	Primer sequence (5′→3′)	Reverse primer	Primer sequence (5′→3′)	T_A (°C)
1	HamMT0kbL1	CATACCCCgTTTATgAgAgCA	HamMT3kbR1	ATAAggggTgACgCACTgTC	61
1	HamMT0kbL1		HamMT3kbR2	gTggTCAgCTgggggATAAg	57
1	HamMT0kbL2	ATgCTAATTTCTCCgTCTCAAT	HamMT3kbR1		57
1	HamMT0kbL2		HamMT3kbR2		57
2	HamMT3kbL1	gCgTCACCCCTTATAgAgCA	HamMT6kbR2	CgAAAATgTAgCgTgTTATTTACACTA	57
2	HamMT3kbL2	CCCCAgCTgACCACAgTTAT	HamMT6kbR1	CAACCgCTTAAAATgAAgTTAgAAg	57

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