FRI-UW-9606
February 1996

DEVELOPMENT OF GENETIC MARKERS FOR CRABS

P. BENTZEN AND P. JENSEN

FINAL REPORT

to

ALASKA DEPARTMENT OF FISH AND GAME
GENETICS LAB
333 RASPBERRY ROAD
ANCHORAGE, AK 99518

FRI-UW-9606
February 1996

FISHERIES RESEARCH INSTITUTE
SCHOOL OF FISHERIES
UNIVERSITY OF WASHINGTON
SEATTLE, WA 98195

DEVELOPMENT OF GENETIC MARKERS FOR CRABS

P. BENTZEN AND P. JENSEN

FINAL REPORT

to

ALASKA DEPARTMENT OF FISH AND GAME
GENETICS LAB
333 RASPBERRY ROAD
ANCHORAGE, AK 99518

Approved

Submitted 2-12-96

Director

CONTENTS

LIST OF FIGURES

LIST OF TABLES

KEYWORDS

Alaska, *Chionoecetes*, Bering Sea, hybrid, king crab, *Paralithodes camtschaticus*, species marker, snow crab, systematics, Tanner crab

INTRODUCTION

Red king crab (*Paralithodes camtschaticus*) and Tanner crab (*Chionoecetes* spp.) support important fisheries in Alaska. There is a need for genetic markers that can be used to identify reproductively isolated stocks of these species for proper management and enforcement of fisheries regulations. In the case of Tanner crab, there is also a need for genetic markers that facilitate the identification of species, since species in this group are known to hybridize (Karinen et al. 1971).

This report describes the first phase of research conducted at the Marine Molecular Biotechnology Laboratory at the University of Washington to identify stock markers for king and Tanner crab, and species markers for Tanner crab. We have evaluated the potential of mitochondrial and nuclear ribosomal DNA sequences for population identification in both groups of crabs, and have developed nuclear and mitochondrial DNA markers that allow the identification of *Chionoecetes* species and their hybrids.

OBJECTIVES

I. Development of rapid DNA extraction methodologies for crab based on frozen tissues.
II. Identification of primers suitable for PCR amplification of mtDNA sequences from crab.
III. Identification of potential DNA-based species markers for *Chionoecetes* spp.
IV. Identification of polymorphic sites in *Paralithodes camtschaticus* mtDNA and preliminary assessment of their utility in analyses of population structure.

Frozen tissues were provided by the Alaska Department of Fish and Game (ADFG, Fig. 1). Muscle tissue was taken from the following:

50 *Paralithodes camtschaticus*, red king crab, Stock A; labeled A1–A50
50 *P. camtschaticus* Stock B; labeled B1–B50
50 *P. camtschaticus* Stock C; labeled C1–C50
25 *Chionoecetes bairdi*, Tanner crab; labeled Cb1–Cb25
25 *C. opilio*, snow crab; labeled Co1–Co25
15 *C. opilio* X *C. bairdi* hybrids; labeled H1–H4, H10, H12–H14, H20–H21, H23–H27.

Gill, heart, and hepatopancreas tissue samples were taken from five individuals from each category above, excluding hybrid *Chionoecetes*.

RESULTS

I. DNA Extraction Methodologies

Standard Phenol:Chloroform Extraction

We used standard phenol:chloroform (P:C) extraction as the starting point in developing a rapid extraction methodology for frozen crab tissues. Standard P:C extraction consists of an overnight

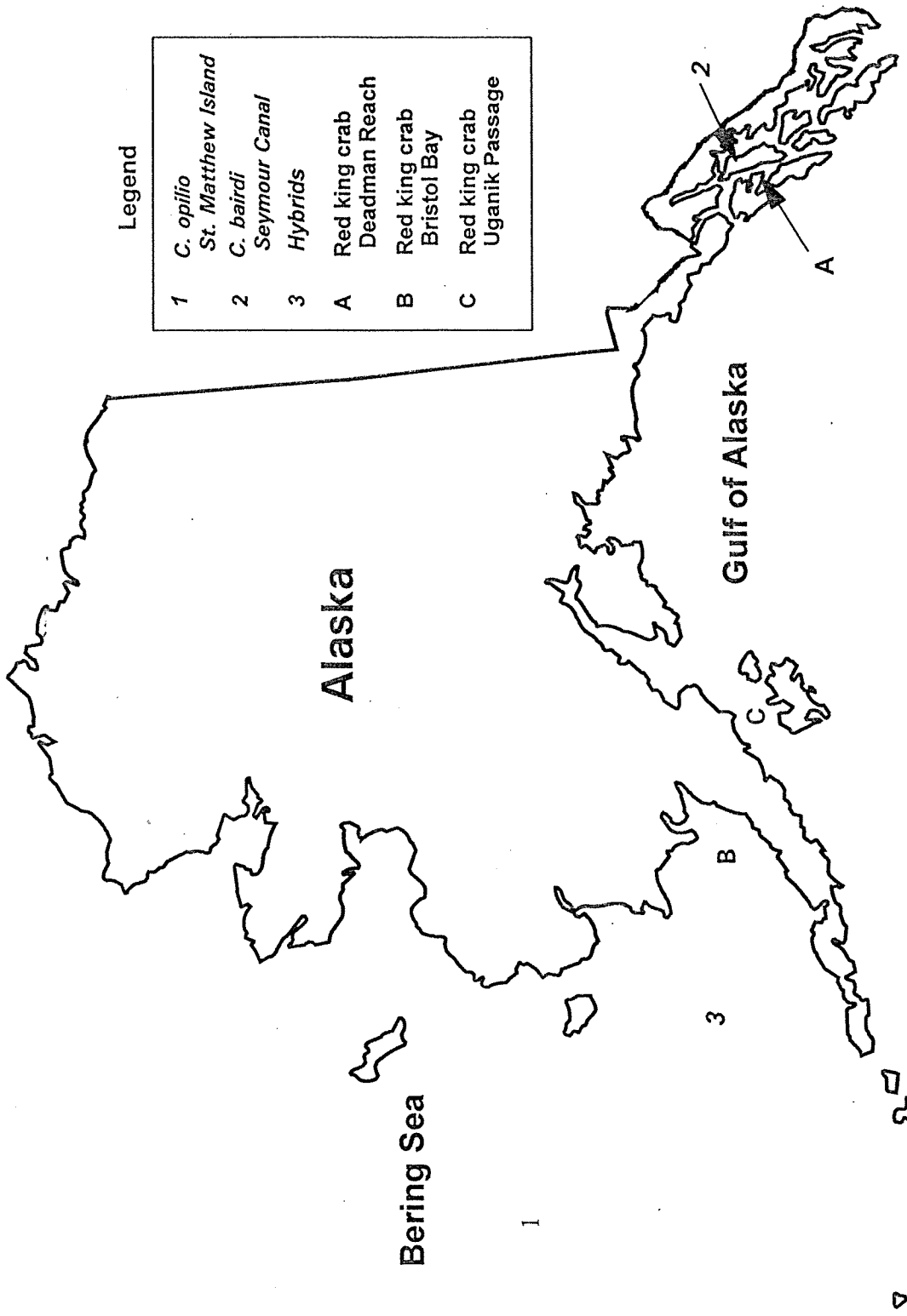


Figure 1. Collection sites for crab tissue samples used in this study. Source: Sue Merkouris, ADFG, Anchorage.

digestion in lysis buffer (see Appendix for recipes of buffers, detailed descriptions of protocols, etc.) followed by two P extractions, two P:C extractions, two C extractions, and precipitation of the resulting genomic DNA in EtOH. An aliquot of the extracted genomic DNA was visualized on 2% agarose gels post stained with ethidium bromide (EtBr) and all DNA extractions were quantified in a spectrophotometer (GeneQuant RNA/DNA Calculator, Pharmacia). Typical yields from ~10 mg of tissue were 500–800 ng/µl (range 30–1700 ng/µl) in a total volume of 100 µl. Because spectrophotometers incorporate degraded DNA and contaminants into the reading of DNA concentration, spectrophotometer readings alone are not reliable indicators of DNA quantity; a sample with a low reading can have more high-quality DNA than a sample with a higher reading. As an extreme example, after extraction of a hepatopancreas sample, photometric quantification gave a concentration of 1.188 µg/µl; however, gel visualization revealed that only degraded DNA was present. Therefore, we visualized all extractions on a gel in addition to quantifying extractions by spectrophotometry.

We compared genomic DNA yields of four tissue types (gill, heart, hepatopancreas, muscle) in *C. bairdi*, *C. opilio*, and *P. camtschaticus* utilizing standard P:C extraction protocol. In red king crab, similar-sized pieces of gill, heart, and muscle tissue yielded DNA of the same quality (as visualized on 2% agarose gels and as PCR templates), with heart tissue yielding ~10% more DNA. In Tanner and snow crab, similar-sized pieces of gill, heart, and muscle tissue yielded DNA of the same quality (as visualized on 2% agarose gels and as PCR templates), with heart tissue yielding only slightly more (~5%) DNA. Hepatopancreas tissues of all three species were difficult to work with and yielded only degraded DNA. Because muscle tissue produces high quality DNA in amounts comparable with gill and heart tissue and can be sampled by non-lethal means, we recommend that tissue samples be taken from muscle.

Quick Phenol:Chloroform Extraction for Crab Tissues

We developed a quick P:C extraction protocol for frozen crab tissue. The standard protocol was followed, but the number of organic extractions was reduced from six to three. In most cases, this protocol performed as well as the standard. However, some samples had a heavier protein precipitate at the interface and those samples required further extractions, in some cases as many organic extractions as in standard P:C extraction. Typically, there was less protein precipitate in king crab tissue extractions than in *Chionoecetes* spp.

The quick P:C protocol yielded genomic DNA of the same quantity and quality (as visualized on 2% agarose gels and as a PCR template) as the standard method, and we used this method for most of the tissue extractions (n = 173 muscle tissue samples).

Phenol:Chloroform Extraction and Serum Separator Tubes

In an attempt to further shorten the time involved in extracting DNA, we performed P:C extractions in serum separator tubes (SST; Vacutainer® Beckton Dickinson). The use of SST eliminated transfer of supernatant to clean tubes; the proteins and organic solvents migrated below the plug during each centrifugation step, leaving the aqueous phase and the DNA above the plug. For crab tissues, the use of SST reduced genomic DNA yields and in some of the subsequent PCRs either

no amplification products (ITS III and IX) or reduced yields of amplification products were produced (co1a and f); 16Sar and 16Sbr yields were unaffected. We do not recommend the use of SST for crab tissue extractions.

Salt Extraction

We used the Puregene salt extraction kit (Gentra Systems, Inc., the same kit that ADFG uses; S. Merkouris, ADFG, Anchorage, pers. comm.) to compare P:C and salt extractions of frozen crab tissue. We followed the manufacturer's instructions (see Appendix), except, since we did not use RNase in P:C extraction, we did not treat the salt extracted tissues with RNase. Salt extraction, when preceded by either an hour or overnight incubation in lysis buffer with PK, resulted in genomic DNA yields that were comparable in quantity and quality with P:C extraction yields in king crab. A 1-h lysis incubation without PK followed by salt extraction resulted in approximately half the yield as that from P:C extraction in king crab. In *C. opilio,* an overnight incubation in lysis buffer with PK followed by salt extraction yielded only half as much genomic DNA as did extraction with P:C; a 1-h incubation without PK followed by salt extraction reduced yield by another 10%.

Rapid Extraction (Lysis Only)

Tissues were minced or ground with a plastic pestle and placed in lysis buffer for 1 h and then spun down. The supernatant was removed to a clean tube and used as template in PCR without further processing. Although this technique performs well for a number of fish species (Paul Bentzen, pers. observ.), in crabs the genomic DNA yield was extremely low and typically could not be visualized on a gel, and the PCR product was extremely faint or nonexistent. Modifications of this technique (increased incubation time, increased concentration of PK, increased amount of tissue, change of lysis buffers, addition of DTT) did not result in an improvement in yield.

II. Identification of PCR primers

Primer sequences (Table 1) were acquired from the literature and either synthesized in the Marine Molecular Biotechnology Laboratory (MMBL), obtained from other investigators, or ordered from reputable supply firms. A number of primer combinations were tested (Table 2) and those yielding product in the expected size range were optimized by raising the annealing temperature (T_A°) until only a sharp, clean (no smears or nonspecific bands) target band was produced; the number of PCR cycles was varied to achieve high yields. If raising the T_A° alone did not result in clean target product, a Mg titration was done at the highest T_A° that had produced target product. PCR profiles were held constant with a 5s warming at 96°C followed by the desired number of cycles of {94°C (10s) TA°C (15s) 72° (30s)}. Amplifications were performed in a Perkin-Elmer 9600 thermal cycler. The PCR mix contained 100 ng template (genomic DNA), 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.0–7.0 mM $MgCl_2$ (depending on primer pair), 0.4 mM each dNTP (1.6 mM total dNTPs), 1.0 unit *Taq* (Promega), and 0.5 μM of each primer

Table 1. Primer sequences and sources. Y = C, T; NA, not available.

Primer	Sequence 5'...3'	Map position	Source
Mitochondrial			
16Sar-L	CGC CTG TTT ATC AAA AAC AT	13,398[a]	Palumbi et al. 1991
16Sbr-H	CCG GTC TGA ACT CAG ATC ACG T	12,887[a]	Palumbi et al. 1991
12SA-L	AAA CTG GGA TTA GAT ACC CCA CTA T	14,612[a]	Palumbi et al. 1991
12SB-H	GAG GGT GAC GGG CGG TGT GT	14,211[a]	Palumbi et al. 1991
12sai-L	AAA CTA GGA TTA GAT ACC CTA TTA T	14,588[a]	Palumbi et al. 1991
12sbi-H	AAG AGC GAC GGG CGA TGT GT	14,214[a]	Palumbi et al. 1991
Co1f-L	CCT GCA GGA GGA GGA GAY CC	2131[a]	Palumbi et al. 1991
Co1a-H	AGT ATA AGC GTC TGG GTA GTC	2791[a]	Palumbi et al. 1991
Co1 j	CAA TAC CTG TGA GTC CTC CTA	1729[b]	Van Syoc 1993
Co1 k	GAG CTC CAG ATA TAG CAT TCC	2536[b]	Van Syoc 1993
Co2a	GGG GCT AAC CAT AGA TTC ATG CC	3682[a]	Palumbi et al. 1991
Co3	AAC ATC TCG TCA TCA TTG A	NA[c]	Bucklin and Stanton 1992
ISOA-2	TTA CGG GCG TAT TTT ACT TG	NA[d]	Brown 1993
cyt *b* L15408	ATA GAC AAA ATC CCA TTC CA	15,408[e]	Irwin et al. 1991
cyt *b* H15915	AAC TGC AGT CAT CTC CGG TTT ACA AGA C	15,915[e]	Irwin et al. 1991
cyt *b* L14724	TGA CTT GAA GAA CCA CCG TTG	14,724[e]	Irwin et al. 1991
ND3 c-gly	GTA CAC GTG ACT TCC AAT CA	NA[f]	L. Park (NMFS, Seattle, pers. comm.)
ND4 c-his	AGA ATC ACA ATC TAA TGT TT	NA[f]	L. Park (NMFS, Seattle, pers. comm.)
ND5	CCA CGG TGG TTC TTC AAG TC	NA[f]	L. Park (NMFS, Seattle, pers. comm.)
ND6	GGA ACC AAA AAC TCT TGG TGC AAC TCC	NA[f]	L. Park (NMFS, Seattle, pers. comm.)
Nuclear			
ITS III	CAC ACC GCC CGT CGC TAC TAC CGA TTG	1640[e]	Hillis and Dixon 1991
ITS VIII	GTG CGT TCG AAG TGT CGA TGA TCA A	85[e]	Hillis and Dixon 1991
ITS IX	AGA CTC CTT GGT CCG TGT TTC AAG AC	1129[e]	Hillis and Dixon 1991

[a]*Drosophila* spp., [b]*Balanus glandula,* [c]*Daphnia* spp. and *Homarus* spp., [d]*Semibalanus balanoides,* [e]mammals, [f]*Gadus morhua*

in a total volume of 25 µl. Positive (*Oncorhynchus mykiss*, rainbow trout, genomic DNA) and negative (reactions without DNA template) controls were always run during PCRs. PCR products were visualized on 2% agarose gels post stained in EtBr. Table 2 also lists the recommended T_A° and [Mg] for the primer pairs that produced clean products; Table 3 lists the sizes of PCR amplified products in crabs.

Chionoecetes

The primer pairs 16Sar/16Sbr, co1a-H/co1f-L, and ITS III/IX reliably produced high yields of clean, sharp target bands in the expected size ranges (Table 3) in *C. bairdi, C. opilio*, and *Chionoecetes* hybrids.

P. camtschaticus

The primer pairs 16Sar/16Sbr and ITS III/IX reliably produced high yields of clean, sharp target bands of the expected size ranges (Table 3). The primers co1a-H and co1f-L produced three

Table 2. Primer pairs tested. Numbers are the annealing temperature followed by [Mg] and number of PCR cycles. NA, not attempted; +, clean and sharp product; —, no product, smears, multiple bands, and/or junk (the wrong product band). Rainbow trout was used as a positive control in PCR.

Primer pairs	Red king crab	*C. bairdi*	*C. opilio*	*Chionoecetes* hybrids	Rainbow trout
Mitochondrial					
16Sar & br	+ 52° [3.0] 25	+ 50° [3.0] 25	+ 50° [3.0] 25	+ 50° [3.0] 25	+
12SA & B	—	—	—	NA	+
12sai & bi	+ 56° [3.0] 25	+ 56° [3.0] 25	+ 56° [3.0] 25	NA	—
12Sbi & IsoA-2	—	—	—	NA	—
IsoA-2 & 12S B	—	—	—	NA	—
IsoA-2 & 16S B	—	—	—	NA	—
12SA & 16Sbr	—	—	—	NA	—
12SA & 12sbi	—	—	—	NA	—
Co1a & f	internal priming	+ 50° [3.0] 25	+ 50° [3.0] 25	+ 50° [3.0] 25	+
Co1a & k	+ 52° [3.0] 26	+ 54° [3.0] 26	+ 54° [3.0] 26	+ 54° [3.0] 26	+
Co1j & k	—	—	—	NA	+
Co1j & f	—	—	—	NA	+
Co2a & Co3	—	+ 56° [3.0] 25	+ 56° [3.0] 25	NA	—
cyt b L15408 & H15915	—	—	—	NA	—
cyt *b* H15915 & L14724	—	—	—	NA	+
ND3 & 4	—	—	—	NA	+
ND5 & 6	—	—	—	NA	+
Nuclear					
ITS III & IX	+ 54° [1.5] 27	+ 60° [1.5] 25	+ 60° [1.5] 25	+ 65° [3.0] 25	—
ITS III & VIII	—	—	—	NA	+

Table 3. Approximate size of PCR amplification products (in kb) in *C. bairdi*, *C. opilio*, and *P. camtschaticus*. NA, not attempted; —, no product, smears, multiple bands, and/or junk (the wrong product band).

Primer pairs	*P. camtschaticus*	*C. bairdi*	*C. opilio*	*Chionoecetes* hybrids
Mitochondrial				
16Sar & br	.54	.56	.56	.56
12Sai & bi	.43	.46	.46	NA
Co1a & f	internal priming	.72	.72	.72
Co1a & k	1.13	1.13	1.13	1.13
Co2a & Co3	—	1.40	1.40	NA
Nuclear				
ITS III & IX	3.0	3.0	3.0	3.0

amplification products in all three stocks of red king crab owing to the presence of an internal priming site.

III. Identification of Potential DNA-Based Species Markers for *Chionoecetes* spp.

To identify species markers in *Chionoecetes*, we employed restriction enzymes on PCR products. The protocols supplied with the enzymes by the manufacturer (Promega) were followed and the digests were visualized on 2% agarose gels post stained in EtBr. All restriction fragment lengths were estimated with the program FragmeNT Analysis on the FluorImager (Molecular Dynamics). To minimize the future cost of assaying large numbers of samples, we visualized the results on 2% agarose gels and chose potential markers based on the ease of scoring within the range of resolution of the gel; potential species markers are denoted by '*' in Table 4. With higher resolution gels, which are more expensive, more of the enzymes would probably reveal potential

Table 4. Restriction enzymes used and number of restriction sites found in preliminary assays of PCR amplified ITS, 16S, and cytochrome oxidase I (cytox) fragments in *C. bairdi* and C. *opilio*. * denotes a potential species marker; ** denotes assay of the fragment in all available tissue samples by the corresponding enzyme. *Cb = C. bairdi; Co = C. opilio*; — = not assayed; N = any base.

Restriction enzyme	Recognition sequence	No. of restriction sites		
		ITS *Cb/Co*	16S *Cb/Co*	cytox *Cb/Co*
*Alu*I	AG↓CT	6/7*	—	3/4**
*Ava*II	G↓G(AT)CC	3/3*	0/0	0/0
*Bst*OI	CC↓(AT)GG GG(AT)↓CC	3/3	—	0/0
*Cfo*I	GCG↓C	6/6*	0/0	0/0
*Dra*I	TTT↓AAA	1/1	1/1	0/0
Eco l CRI	GAG↓CTC	1/1	—	—
*Eco*RI	G↓AATTC	1/1	—	0/0
Eco RV	GAT↓ATC	—	0/0	—
Hae II	(AG)GCGC↓(TC)	4/4	—	0/0
*Hae*III	GG↓CC	7/7	—	0/0
*Hin*fI	G↓ANTC	5/6*	1/0**	1/1
*Mbo*I	↓GATC	4/4	—	0/0
*Msp*I	C↓CGG	6/5**	—	1/1
*Nci*I	CC↓(CG)GG	7/5**	—	0/0
*Rsa*I	GT↓AC	5/5*	—	0/0
*Sau*3AI	↓GATC	3/3	—	0/0
*Taq*I	T↓CGA	5/5	—	0/0
*Vsp*I	AT↓TAAT	—	1/1	—

species markers, some of the single bands would resolve as two bands, and some of the homologous bands would likely differ in length by one to a few base pairs. Bands below 200 base pairs (bp) were not always resolved depending on the number of restriction sites present (the more sites the lower staining intensity of each resulting band), the size of the band (the smaller the band the lower the staining intensity), the amount of PCR product digested, the run time of the gel, etc.

ITS

We assayed the ITS fragment for restriction sites with 16 restriction enzymes (Table 4; Fig. 2a, b) in *C. bairdi* and *C. opilio* to identify potential species markers. On the basis of ease of scoring, we selected two of the enzymes that had produced species differences, *Msp*I and *Nci*I, as useful candidates for species markers. All 25 *C. bairdi* and 25 *C. opilio* samples were assayed with *Msp*I and *Nci*I. Restriction of the ITS fragment in *C. bairdi* by *Msp*I produced seven bands in C. *bairdi* and six bands in *C. opilio*; two of the bands were present in only *C. bairdi* and a third in only *C. opilio* (Table 5). Digestion with *Nci*I produced eight bands in *C. bairdi* and six in *C. opilio*; two of the bands were present in only *C. bairdi* and a third in only *C. opilio* (Table 5). No intraspecific differences within either species were detected.

Cytochrome oxidase I

We assayed the cytochrome oxidase I fragment for restriction sites with 15 restriction enzymes (Table 4) in *C. bairdi* and *C. opilio*; only one of the enzymes, *Alu*I, produced a potential species marker. All 25 *C. bairdi* and 25 *C. opilio* samples were assayed with *Alu*I. Restriction of the cytochrome oxidase fragment in *C. bairdi* with *Alu*I produced four bands; in *C. opilio*, five bands were produced (Table 6; Fig. 3). One band, 0.25 kb, is present only in *C. bairdi*; a second band, 0.21 kb, is present only in *C. opilio* but often is not adequately resolved from the 0.20 kb band on a 2% agarose gel. Therefore, the 0.20 kb fragment may not be a useful marker. No intraspecific differences within either species were detected.

16S

We sequenced ~465 base pairs of the 16S rRNA gene amplified with the 16Sar and br primers in three individuals each of *C. bairdi* and *C. opilio* (Table 7). On the basis of preliminary sequence data we used GCG to search for restriction sites in 16S; six enzymes were selected: *Ava*II, *Cfo*I, *Dra*I, *Eco*RV, *Hinf*I, and *Vsp*I. One enzyme, *Hinf*I, produced 2 bands (0.36 and 0.22 kb) in *C. bairdi* but did not cut *C. opilio* 16S (Tables 4 and 8; Fig. 4); the other five enzymes did not produce potential species markers (Tables 4 and 8). The selection of *Ava*II, *Cfo*I, *Dra*I, *Eco*RV, and *Vsp*I was based on ambiguous sites in the sequencing data that have since been resolved. No intraspecific differences within either species were detected with restriction digests.

The 16S sequences were aligned with CLUSTAL (Applied Biosystem, Inc.'s Sequence Navigator™). Three interspecific differences in the sequences, all CT substitutions, were detected (Table 7). The second substitution, at the 166th base sequenced, is within the *Hinf*I restriction site in C. *bairdi*. No intraspecific differences in 16S sequences were found.

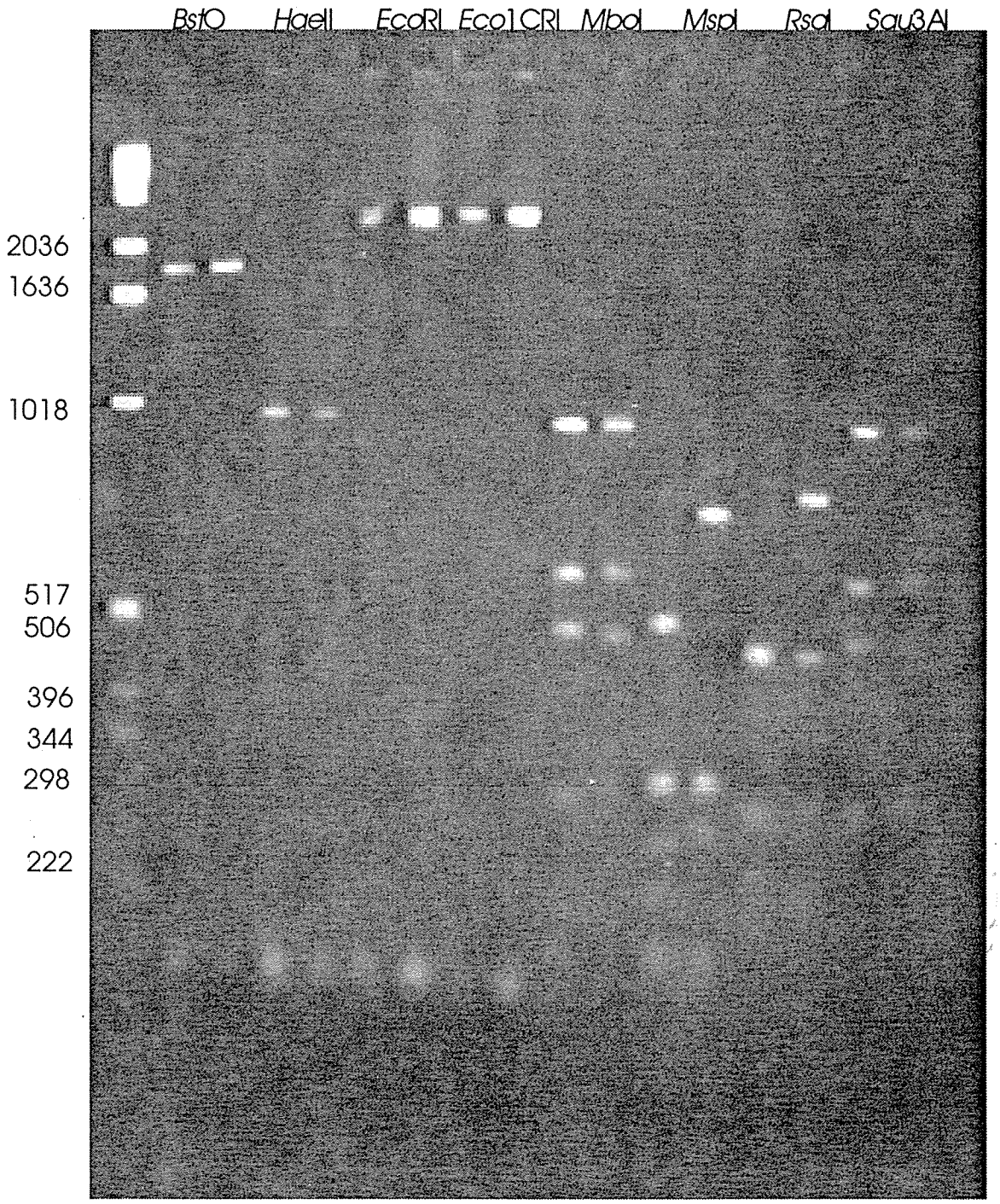


Figure 2a. Restriction enzyme digests of paired *C. bairdi* (even-numbered lanes) and *C. opilio* (odd-numbered lanes) PCR amplified ITS fragments, e. g., lanes 2 and 3 are *Bst*O digests of *C. bairdi* and *C. opilio* ITS, respectively, lanes 4 and 5 are *Hae*II digests of *C. bairdi* and *C. opilio* ITS, respectively, etc. Lane 1, 1 kb ladder.

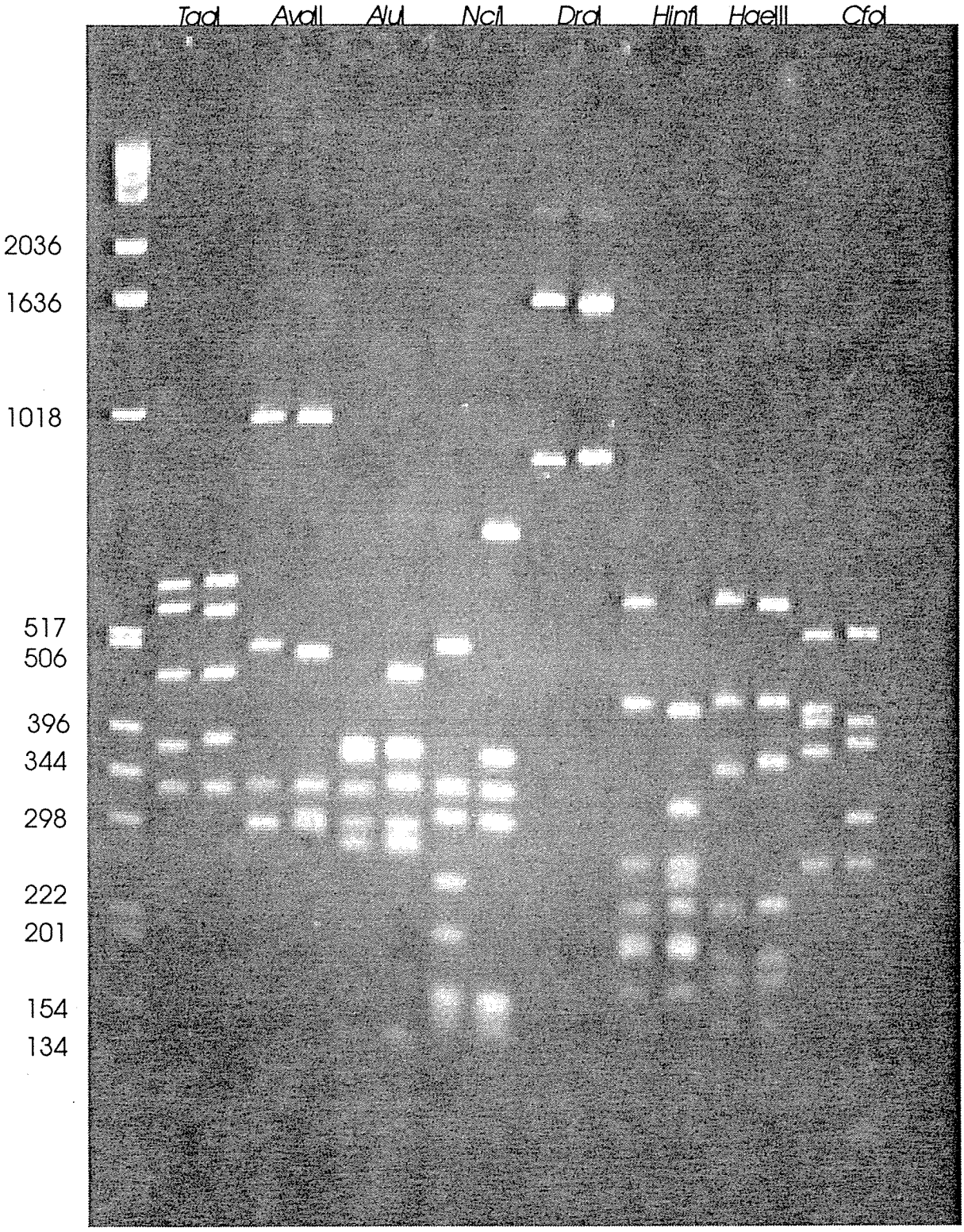


Figure 2b. Restriction enzyme digests of paired *C. bairdi* (even-numbered lanes) and *C. opilio* (odd-numbered lanes) PCR amplified ITS fragments; labeling as in Fig 2a.

Table 5. Estimated sizes (in kb) of restriction enzyme-digested PCR amplified ITS fragment. Potential species markers are bold; enzymes used to restrict all tissue samples denoted by *. Fragments below 0.02 kb were not regularly resolved.

Restriction enzyme	Restriction fragment sizes (bp) of ITS	
	C. bairdi	*C. opilio*
*Alu*I	0.37, 0.33, 0.30, 0.28, 0.14, 0.09, 0.08	**0.47**, 0.37, 0.33, 0.30, 0.28, 0.17, 0.085, 0.080
*Ava*II	1.03, 0.51, 0.33, 0.30	1.03, 0.50, 0.33, 0.30
*Bst*OI	1.83, 0.39, 0.15, 0.12	1.85, 0.39, 0.15, 0.12
*Cfo*I	0.52, 0.**42**, 0.40, 0.37, 0.27, 0.11, 0.08	0.53, 0.41, 0.39, 0.**31,** 0.27, 0.11, 0.09
*Dra*I	1.66, .90	1.66, .90
*Eco*l CRI	2.78, 0.14	2.78, 0.14
*Eco*RI	2.61, 0.15	2.61, 0.15
*Hae*II	0.98, 0.46, 0.43, 0.31, 0.15	0.98, 0.47, 0.43, 0.30, 0.15
*Hae*III	0.60, 0.44, 0.35, 0.23, 0.19, 0.17, 0.15, 0.11	0.60, 0.44, 0.36, 0.23, 0.19, 0.17, 0.15, 0.11
*Hinf*I	**0.60**, 0.43, 0.26, 0.23, 0.19, 0.17	0.42, **0.32,** 0.26, 0.25, 0.23, 0.19, 0.17
*Mbo*I	0.96, 0.58, 0.48, 0.29, 0.14	0.97, 0.58, 0.48, 0.30, 0.14
*Msp*I*	**0.51**, 0.30, 0.28, **0.21**, 0.17, 0.15, 0.11	**0.70,** 0.30, 0.26, 0.17, 0.15, 0.11
*Nci*I*	**0.51**, 0.33, 0.30, **0.25, 0.20**, 0.16, 0.15, 0.09	**0.71, 0.36**, 0.33, 0.30, 0.16, 0.15
*Rsa*I	0.46, 0.27, 0.21, 0.20, **0.18**, 0.14	**0.75**, 0.45, 0.28, 0.22, 0.20, 0.13
*Sau*3AI	0.94, 0.55, 0.47, 0.27	0.95, 0.56, 0.47, 0.28
*Taq*I	0.60, 0.56, 0.46, 0.37, 0.33, 0.09	0.60, 0.55, 0.46, 0.38, 0.33, 0.09

Table 6. Estimated sizes (in kb) of restriction enzyme-digested PCR amplified (with col a and col f) cytochrome oxidase I fragment. Potential species markers are bold; enzymes used to restrict all tissue samples denoted by *. Fragments below 0.01 kb were not regularly resolved.

Restriction enzyme	Restriction fragment sizes (bp) of cytochrome oxidase I	
	C. bairdi	*C. opilio*
*Alu*I*	**0.25**, 0.20, 0.17, 0.08	0.21, 0.20, 0.17, 0.08, 0.05
*Hinf*I	0.55, 0.06	0.56, 0.07
*Msp*I	0.57, 0.12	0.57, 0.12

Hybrid Chionoecetes

16S, cytochrome oxidase I, and ITS were amplified from all 15 of the hybrid *Chionoecetes* samples as described for *C. bairdi* and *C. opilio* (Table 2); amplification products were of the same approximate sizes as those from the putative parent species (Table 3). The 16S and cytochrome oxidase fragments were assayed with *Hinf*I and *Alu*I, respectively. Samples H1 and H23 revealed the same *Hinf*I and *Alu*I restriction sites as *C. bairdi* while the other 13 samples displayed the same digestion bands as *C. opilio* (Table 5; Fig. 3). This suggests that the mtDNA of H1 and H23 was inherited from *C. bairdi* females and the mtDNA of the 13 other hybrid crab from *C. opilio* females implying that most hybrids are the result of matings between female *C. opilio* and male *C. bairdi*. The hybrid ITS fragments were restricted with *Nci*I (Fig. 5) and *Msp*I (Fig. 6). If all of the hybrids were F1s, then all 15 hybrids should have had all of the parental species restriction sites in the ITS fragments. All but individuals H10 and H13 displayed all of the restriction bands from both parental species. Individuals H10 and H13 were lacking the

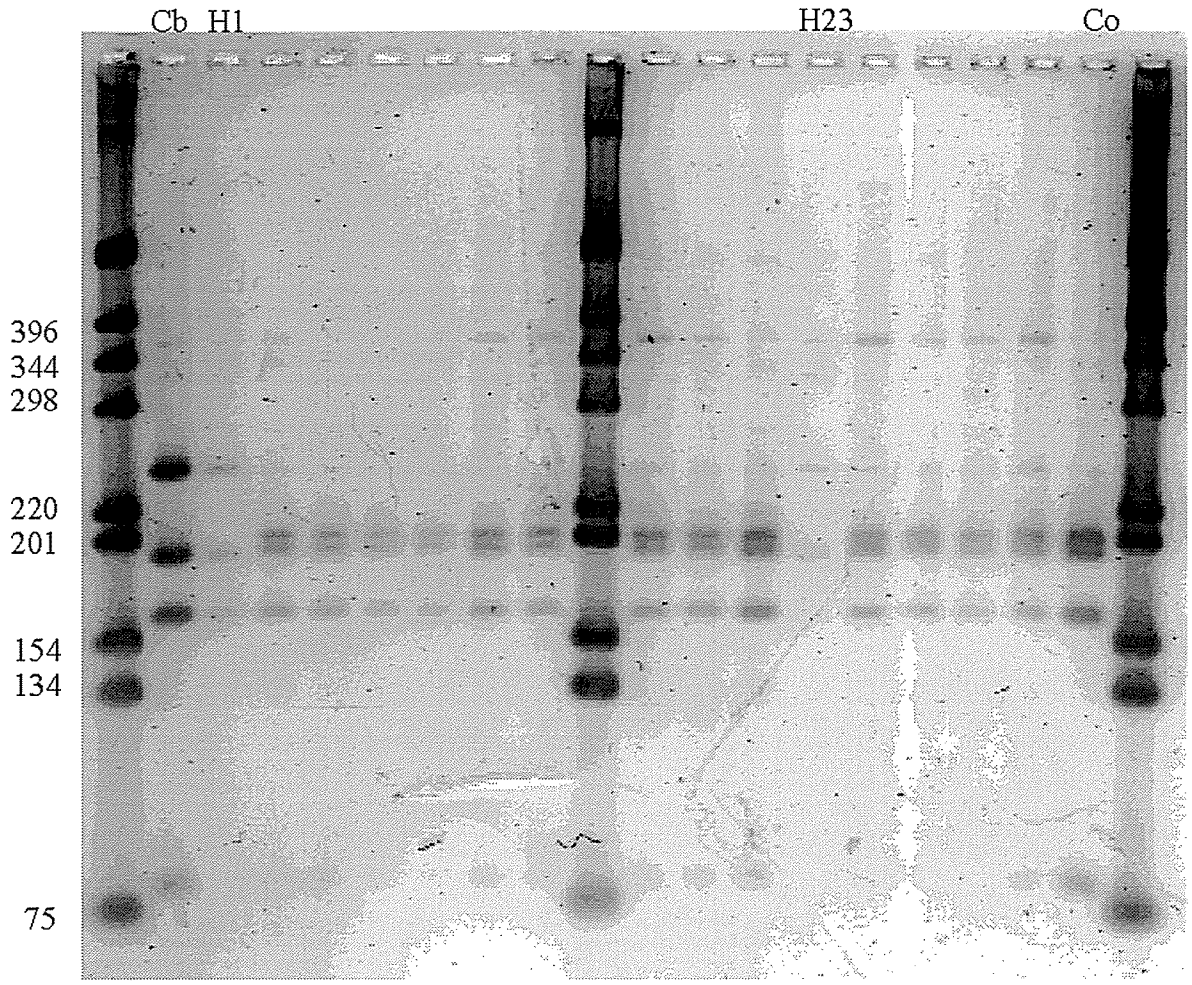
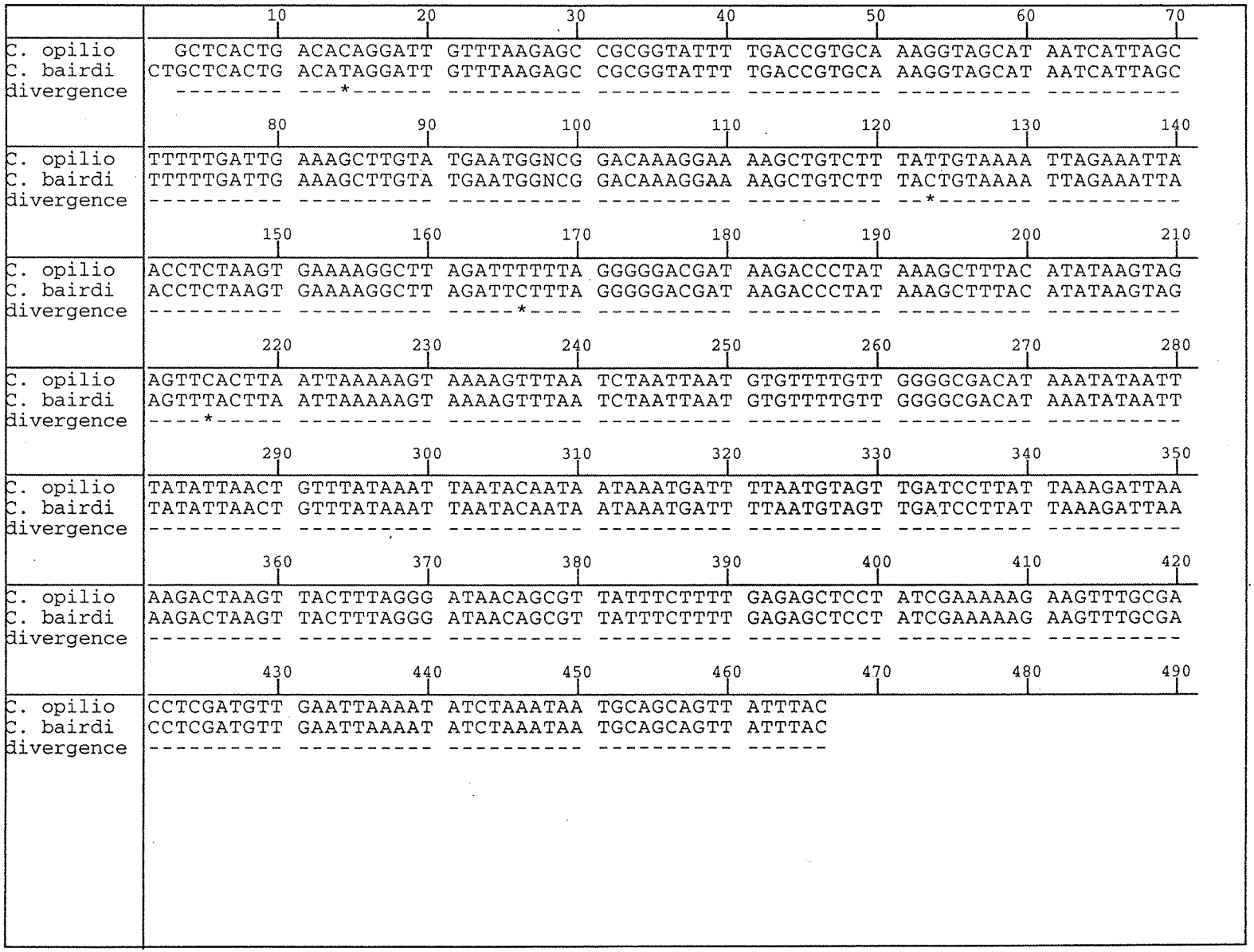


Figure 3. *Alu*I digest of PCR amplified cytochrome oxidase I in hybrid *Chionoecetes*. Lanes 1, 10, and 20, 1 kb ladder; lane 2, *C. bairdi*; lane 19, *C. opilio*.

unique *C. bairdi* bands (0.51, 0.25, and 0.20 kb in *Nci*I digests and 0.51 and 0.21 kb in *Msp*I digests) suggesting that they are back-crosses with *C. opilio* or misidentified *C. opilio*. In all hybrid individuals except H10 and H13, the *Nci*I digest of ITS also produced a band of ~ 0.73 kb. A band of this molecular weight appeared in some *C. bairdi* digests (Fig. 5 and other data not shown). When present, it appeared in substoichiometric amounts, suggesting that it was a partial digestion product, possibly of the 0.51 and 0.20 kb bands unique to *C. bairdi*. Alternatively, the presence of the band in all the hybrid digests, and the absence of other obvious 'partials' in these digests suggests that the 0.73 kb band could be something other than an artifact of incomplete digestion. The organization of the nuclear rRNA genes in crabs is unknown; therefore, the inheritance patterns are not known. The 0.73 kb band may represent heterozygosity in this multicopy gene. Unless supported by future processing that increases sample sizes, the 0.73 kb fragment should not be used as a marker.



	10	20	30	40	50	60	70
C. opilio	GCTCACTG	ACACAGGATT	GTTTAAGAGC	CGCGGTATTT	TGACCGTGCA	AAGGTAGCAT	AATCATTAGC
C. bairdi	CTGCTCACTG	ACATAGGATT	GTTTAAGAGC	CGCGGTATTT	TGACCGTGCA	AAGGTAGCAT	AATCATTAGC
divergence	--------	---*------	----------	----------	----------	----------	----------
	80	90	100	110	120	130	140
C. opilio	TTTTTGATTG	AAAGCTTGTA	TGAATGGNCG	GACAAAGGAA	AAGCTGTCTT	TATTGTAAAA	TTAGAAATTA
C. bairdi	TTTTTGATTG	AAAGCTTGTA	TGAATGGNCG	GACAAAGGAA	AAGCTGTCTT	TACTGTAAAA	TTAGAAATTA
divergence	----------	----------	----------	----------	----------	--*-------	----------
	150	160	170	180	190	200	210
C. opilio	ACCTCTAAGT	GAAAAGGCTT	AGATTTTTTA	GGGGGACGAT	AAGACCCTAT	AAAGCTTTAC	ATATAAGTAG
C. bairdi	ACCTCTAAGT	GAAAAGGCTT	AGATTCTTTA	GGGGGACGAT	AAGACCCTAT	AAAGCTTTAC	ATATAAGTAG
divergence	----------	----------	-----*----	----------	----------	----------	----------
	220	230	240	250	260	270	280
C. opilio	AGTTCACTTA	ATTAAAAAGT	AAAAGTTTAA	TCTAATTAAT	GTGTTTTGTT	GGGGCGACAT	AAATATAATT
C. bairdi	AGTTTACTTA	ATTAAAAAGT	AAAAGTTTAA	TCTAATTAAT	GTGTTTTGTT	GGGGCGACAT	AAATATAATT
divergence	----*-----	----------	----------	----------	----------	----------	----------
	290	300	310	320	330	340	350
C. opilio	TATATTAACT	GTTTATAAAT	TAATACAATA	ATAAATGATT	TTAATGTAGT	TGATCCTTAT	TAAAGATTAA
C. bairdi	TATATTAACT	GTTTATAAAT	TAATACAATA	ATAAATGATT	TTAATGTAGT	TGATCCTTAT	TAAAGATTAA
divergence	----------	----------	----------	----------	----------	----------	----------
	360	370	380	390	400	410	420
C. opilio	AAGACTAAGT	TACTTTAGGG	ATAACAGCGT	TATTTCTTTT	GAGAGCTCCT	ATCGAAAAAG	AAGTTTGCGA
C. bairdi	AAGACTAAGT	TACTTTAGGG	ATAACAGCGT	TATTTCTTTT	GAGAGCTCCT	ATCGAAAAAG	AAGTTTGCGA
divergence	----------	----------	----------	----------	----------	----------	----------
	430	440	450	460	470	480	490
C. opilio	CCTCGATGTT	GAATTAAAAT	ATCTAAATAA	TGCAGCAGTT	ATTTAC		
C. bairdi	CCTCGATGTT	GAATTAAAAT	ATCTAAATAA	TGCAGCAGTT	ATTTAC		
divergence	----------	----------	----------	----------	------		

Table 7. Alignment of 16S gene sequence form *C. opilio* and *C. bairdi*. * denotes an interspecific difference. N = unresolved site.

Table 8. Estimated sizes (in kb) of restriction enzyme-digested PCR amplified (with 16Sar and 16Sbr) 16S fragment. Potential species markers are bold; enzymes used to restrict all tissue samples denoted by *. Fragments below 0.01 kb were not regularly resolved. — = no restriction sites detected.

Restriction enzyme	Restriction fragment sizes (bp) of 16S	
	C. bairdi	*C. opilio*
*Dra*I	0.53	0.53
*Hinf*I*	**0.36, 0.22**	—
*Vsp*I	0.30, 0.22	0.30, 0.22

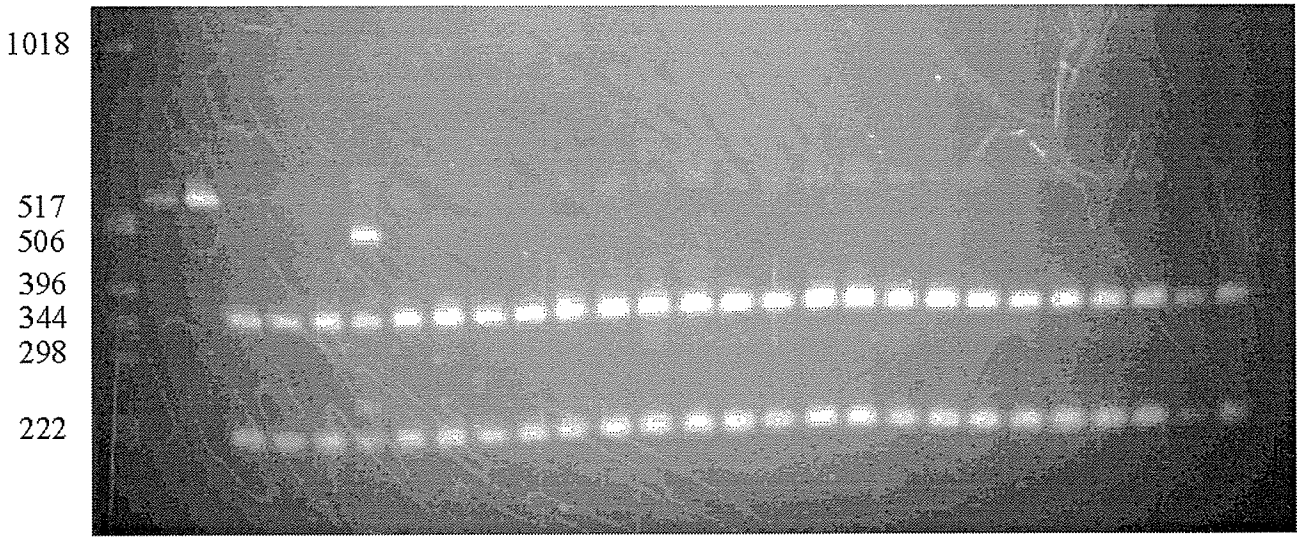


Figure 4. *Hinf*I digest of PCR amplified 16S from all 25 samples of *C. bairdi*. Lane 7 is an incomplete digest. Lane 1, 1 kb ladder; lane 2 undigested *C. bairdi* 16S; lane 3, 'digested' *C. opilio* 16S (no restriction sites).

IV. Identification of Polymorphic Sites in *Paralithodes camtschaticus* mtDNA and Preliminary Assessment of Their Utility in Analyses of Population Structure

ITS

A preliminary assay of the ITS amplified fragment from stocks A, B, and C with 12 restriction enzymes showed no differences between stocks (Table 9); therefore, digests of all tissue samples were not carried out. Restriction site information is summarized in Table 9.

Cytochrome Oxidase I

Because of the presence of an internal priming site in the cytochrome oxidase gene, restriction enzyme surveys were not performed and sequencing could not be done.

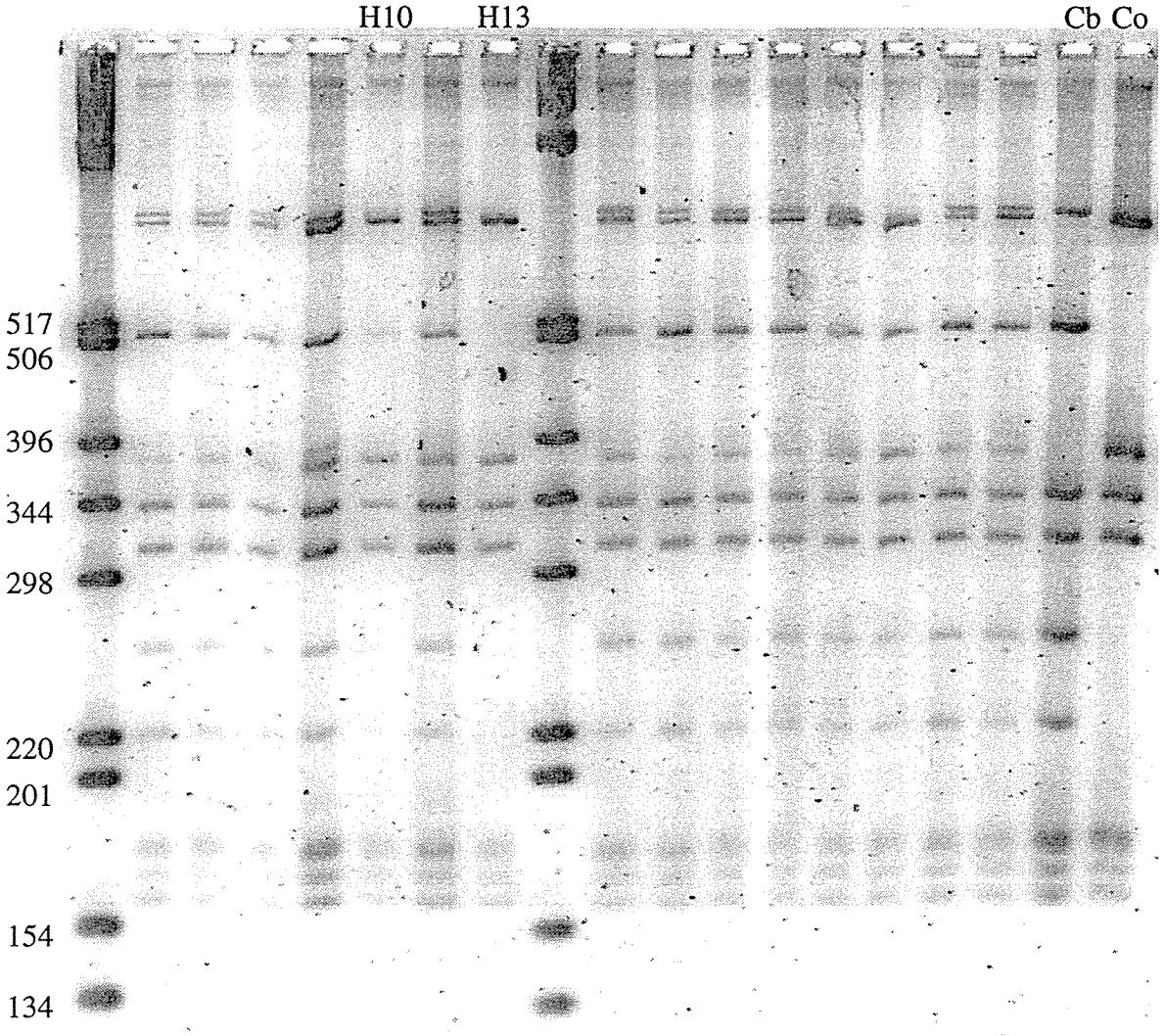


Figure 5. *Nci*I digest of PCR amplified ITS in hybrid *Chionoecetes.* Samples H10 and H13 are possible back-crosses with *C. opilio*. The largest band in the *C. bairdi* lane (18), and in all of the putative hybrids except H10 and H13, may be an incomplete digestion product. See Hybrid *Chionoecetes,* pg. 11 for further details. Lanes 1 and 9, 1 kb ladder; lane 18, *C. bairdi*; lane 19, digested *C. opilio* ITS.

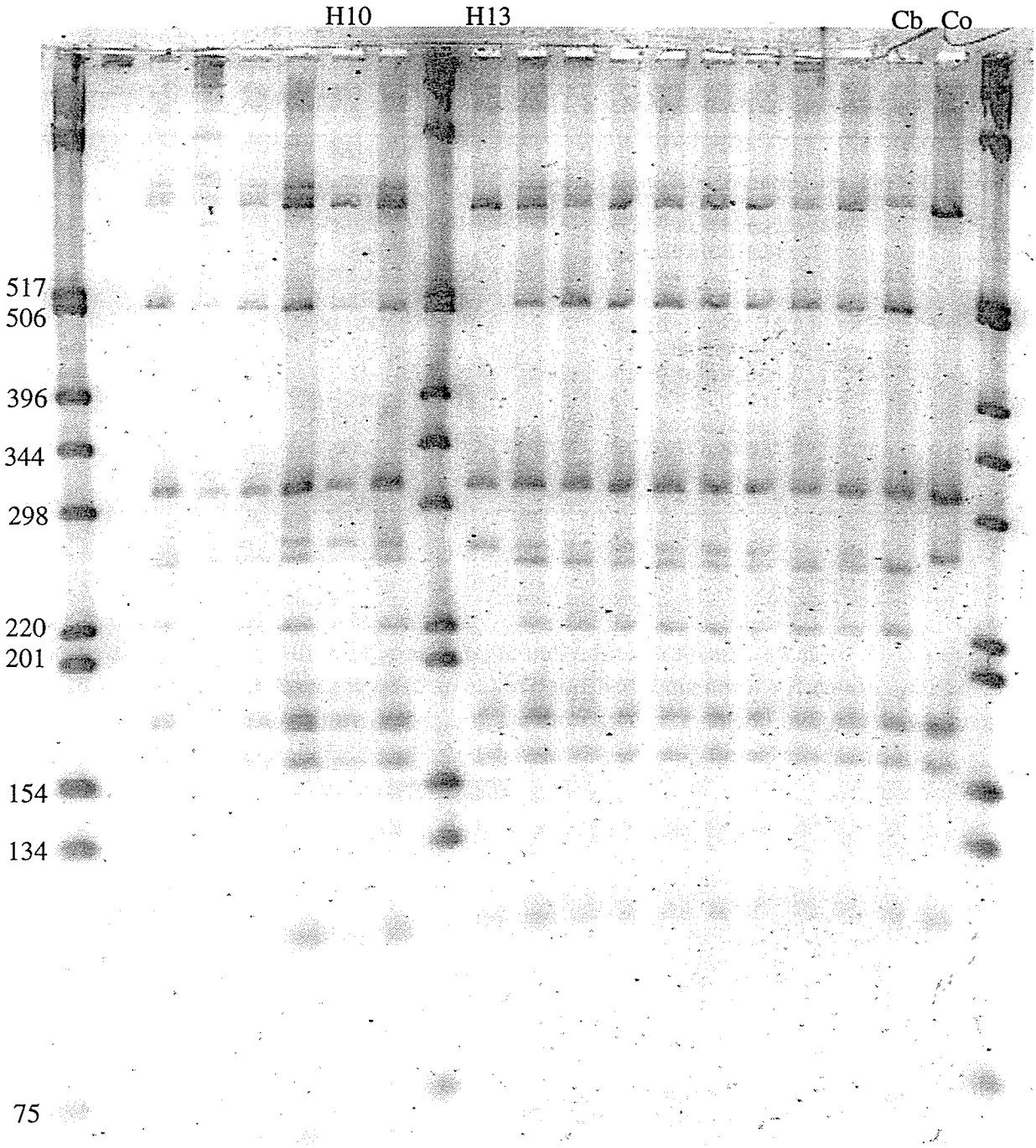


Figure 6. *Msp*I digest of PCR amplified ITS in hybrid *Chionoecetes*. Samples H10 and H13 are possible back-crosses with *C. opilio*. Lanes 1, 11, and 23, 1 kb ladder; lane 21, digested *C. bairdi* ITS; lane 22, digested *C. opilio* ITS.

Table 9. Restriction enzymes used in preliminary assays of PCR amplified ITS fragment and resulting digestion band sizes (kb) in *P. camtschaticus*. No differences between the three stocks, A, B, C, were found. N = any base.

Restriction enzyme	Recognition sequence	Restriction fragment sizes (kb) of ITS
*Alu*I	AG ↓ CT	1.08, 0.40, 0.37, 0.32, 0.24
*Ava*II	G ↓ G(AT)CC	0.94, 0.90, 0.55, 0.24
*Dra*I	TTT ↓ AAA	2.65, 0.41
*Hae*II	(AG)GCGC ↓ (TC)	1.40, 1.05, 0.27
*Hae*III	GG ↓ CC	0.51, 0.45, 0.40, 0.18, 0.16, 0.15
*Hinf*I	G ↓ ANTC	0.75, 0.60, 0.56
*Mbo*I	↓ GATC	1.35, 0.52, 0.39, 0.25, 0.17
*Msp*I	C ↓ CGG	1.85, 0.49, 0.17, 0.15
*Nci*I	CC ↓ (CG)GG	1.93, 0.55, 0.32, 0.25
*Rsa*I	GT ↓ AC	0.61, 0.54, 0.36, 0.33, 0.28, 0.20, 0.17, 0.15
*Sau*3AI	↓ GATC	1.37, 0.54, 0.41, 0.27, 0.18
*Taq*I	T ↓ CGA	1.23, 0.68, 0.48, 0.39

16S

We sequenced ~500 bp in the 16S gene in three individuals from each stock of *P. camtschaticus*. The 16S sequences were identical to published sequence for *P. camtschaticus* (Cunningham et al. 1992); no differences among the three stocks or among individuals within a stock were found. Because sequencing revealed that there were no differences among stocks, restriction enzyme assays were not performed.

LITERATURE CITED

Brown, A. F. 1993. The D-loop of barnacles. Newslet. Crust. Mol. Tech. 4:3.

Bucklin, A. and D. Stanton. 1992. Amplification primers for copepods from *Daphnia* sequence data. Newslet. Crust. Mol. Tech. 1:2-3.

Cunningham, C. W., N. W. Blackstone, and L. W. Buss. 1992. Evolution of king crabs from hermit crab ancestors. Nature 355:539-542.

Hillis, D. M. and M. T. Dixon. 1991. Ribosomal RNA: molecular evolution and phylogenetic inference. Quart. Rev. Biol. 66:411-453.

Irwin, D. M., Kocher, T. D., and A. C. Wilson. 1991. Evolution of the cytochrome *b* gene of mammals. J. Molec. Evol. 32:128-144.

Karinen, J.F., and D.T. Hoopes. 1971. Occurrence of Tanner crabs (*Chionoecetes* sp.) in the eastern Bering Sea with characteristics intermediate between *C. bairdi* and *C. opilio*. Proc. Natl. Shelfish Assoc. 61:8-9

Palumbi, S. R., A. Martin, S. Romano, W. O. McMillan, L. Stice, and G. Grabowski. 1991. The Simple Fool's Guide to PCR, Version 2. University of Hawaii Zoology Dept., Honolulu, HI.

Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: A laboratory manual*, volumes I, II, and III. 2nd ed. Cold Spring Harbor Laboratory Press, New York.

Van Syoc, B. 1993. PCR primers for the COI gene in barnacle mtDNA. Newslet. Crust. Mol. Tech. 4:1-2.

APPENDIX

The Appendix contains protocols developed in this study and recipes that can not be found in Sambrook et al. 1989.

RECIPES

DEB (DNA Extraction Buffer)
1 ml 5M NaCl
10 ml 20% SDS
86 ml ddH_2O
1 ml 1M Tris (pH 8.0)
2 ml 0.5 M EDTA

TE (pH 8.0)
10mM Tris-HCl (pH 8.0)
1 mM EDTA (pH 8.0)
autoclave

1M DTT (DL-dithiothreitol; mw = 154.2)
1.545 g DTT
10.0 ml 0.01 M sodium acetate (pH 5.2)
filter sterilize; aliquot; store @ -20°C

PROCEDURES

Standard phenol:chloroform extraction

1. Mince approximately 10 mg tissue with razor blade (or carefully grind with pestle in a 'large' microcentrifuge tube that contains the DEB — below).
2. Place minced tissue sample in 0.5 ml DEB, 20 µl 1M DTT; 10 µl Proteinase K [10mg/ml] in a 1.6 ml microcentrifuge tube and incubate overnight at 65°C.
3. Add equal volume (0.5 ml) of equilibrated phenol (P).
4. Vortex on highest setting for 30s.
5. Centrifuge 5 min. @ 6,000 rpm in table top microcentrifuge.
6. Remove aqueous phase (supernatant) to clean tube. Transfer as little material from the interface with the supernatant as possible, but it is better to transfer some material from the interface than to leave behind supernatant.
7. Repeat steps 3-5.
8. If the supernatant is clear, go to step 9; if not clear, repeat steps 3-6 until supernatant is clear.

9. Add 0.25 ml 24C:1 IAA (C = chloroform; IAA = isoamyl alcohol) and 0.25 ml P to supernatant.
10. Vortex on highest setting for 30s.
11. Centrifuge 5 min. @ 6,000 rpm in table top microcentrifuge.
12. Remove aqueous phase (supernatant) to clean tube. Transfer as little material from the interface with the supernatant as possible, but it is better to transfer some material from the interface than to leave behind supernatant.
13. Repeat steps 9-12.
14. Add 0.5 ml C:I (24C:1 IAA) to supernatant.
15. Vortex on highest setting for 30s.
16. Centrifuge 5 min. @ 6,000 rpm in table top microcentrifuge.
17. Remove aqueous phase (supernatant) to clean tube. Transfer as little material from the interface with the supernatant as possible, but it is better to transfer some material from the interface than to leave behind supernatant.
18. Repeat steps 14-17, but when transferring the supernatant to a clean tube, **do not** transfer any of the material from the interface.
19. Add 1.0 ml ice cold (-20°C) 100% EtOH to supernatant, invert a few times, and place at -20°C overnight, or for at least 1 h.
20. Centrifuge at 14,000 rpm for 30 min.; note the position of tube/cap hinge in the centrifuge.
21. Carefully pour off (or pipette off) the EtOH, watching to make sure the pellet is not poured out too. (If the pellet is too small to see, you will know its location by the position of the tube in the centrifuge.)
22. Add 1.0 ml 70% EtOH, invert the tube a few times, and spin for 5-10 min. at 14,000 rpm; note the position of tube/cap hinge.
23. **Carefully** pipette off as much EtOH as possible; the pellet will be lose in the tube.
24. Allow the pellet to air dry at room °T or dry in the speed vac (do not overdry in the speed vac).
25. Rehydrate the pellet with 100 µl TE; place on shaker table (rm. °T to 37°C is OK) for 1-2 h.
26. To check the quality of the DNA, run 1 µl of each extraction on a 2% agarose gel. Post stain in EtBr [0.5 µg/ml] for 30-45 min.
27. Quantify the DNA with spectrophotometer.
28. Dilute some of the DNA to make aliquots at a concentration of 100 ng/µl (for PCR working stocks). Check the dilution of the aliquots on the spec. Minimize freeze/thaw events (make the volume of the aliquots 'small').
29. Store at -20°C. (Can keep at 4°C if using the same tube frequently, but the DNA will degrade more quickly than if it is stored at -20°C.)

Quick phenol:chloroform extraction for crab tissues

Follow the standard protocol, except do only one P:C:IAA (0.25 ml P: 0.24 ml C: 1.0 ml IAA) followed by two C:IAA extractions. Note: some crab samples had a heavy precipitate at the interface and required a second P:C extraction; if the interface is especially thick, then one or two P extractions, followed by P:C and then P:C:IAA may be necessary.

The quick P:C yielded genomic DNA of the same quantity and quality (as visualized on 2% agarose gels and as a PCR template) as the standard method.

PCR

All ingredients were kept on ice until placed in the PCR machine.

PCR mix:

100 ng template (genomic DNA)

50 mM KCl

10 mM Tris-HCl (pH 8.3)

1.0–7.0 mM $MgCl_2$ (depending on primer pair)

0.4 mM each dNTP (1.6 mM total dNTPs)

1.0 unit *Taq* (Promega)

0.5 µM of each primer

total volume = 25 µl.

PCR cocktails, excluding the templates, can be mixed in one tube and then aliquoted into the individual PCR tubes (which contain template).

*For negative controls, replace the volume of template with an equal volume of ddH_2O.

*For positive controls, any template that amplifies with the primers being used is satisfactory; *Oncorhynchus mykiss*, rainbow trout, genomic DNA works with almost all of the primer pairs we tested.

Load 5 µl of each PCR product (including neg. and pos. controls) and the appropriate amount of 1 kb ladder (follow supplier's instructions) in loading buffer onto a 2% agarose gel; post stain the gel in EtBr [0.5 µg/ml].

Restriction Digests

Follow the manufacturer's instructions as to proper buffer and incubating temperature for each restriction enzyme. When an enzyme is used for the first time, that is, you don't know how many fragments will be produced by the digest, you will have to guesstimate how much PCR product to use. Look at your PCR product on a gel and try to decide how many bands that product could be split into before you would no longer be able to see it on a gel. If you think you may get a large number of fragments (because your enzyme is a 4-cutter and/or you are restricting a large

fragment) you need more PCR product than if you think you will get only a few fragments. After a preliminary digest, you will know how many fragments there will be and can adjust the amount of PCR product accordingly.

Make the wells in your gel deep enough to hold the entire digest + loading buffer.

You may want to run several ladders (1 on each end and 1 in the middle) on the gel to correct for 'smiles' for accurate sizing.

Sequencing

Clean PCR products are essential for automated sequencing; do not try to sequence PCR products that are anything but sharp and clean when visualized on a gel. Follow the instruction manual supplied with the automated sequencer, EXCEPT, you will probably have to determine the optimal amount of PCR product to put into a sequencing reaction. For example, we have found that 300 ng of crab template should be used when sequencing 16S, not 1/3 of a PCR reaction as the manual states.