

Mitochondrial DNA markers to identify commercial spiny lobster species (*Panulirus* spp.) from the Pacific coast of Mexico: an application on phyllosoma larvae

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Molecular markers based on mitochondrial DNA (mtDNA) are extensively used to study genetic relationships. mtDNA has been used in phylogenetic studies to understand the evolutionary history of species because it is maternally inherited and is not subject to genetic recombination (Gyllensten et al., 1991). The high mutation rate of mtDNA makes it a useful tool for differentiating between closely related species (Brown et al., 1979)—a tool that is especially important when significant variations occur between species, but not within species (Hill et al., 2001; Blair et al., 2006; Chow et al., 2006a).

Species-level identification, based on molecular markers, can be very useful when morphological features alone do not provide sufficient differentiation or when only part of an organism is recovered. In fact, a few authors have successfully applied genetic markers for species identification based on remains recovered from fecal samples, parts of specimens, processed products, or larval and juvenile forms (Chan et al., 2003; Purcell et al., 2004; Hsieh et al., 2007).

Three commercial lobster species inhabit the Pacific coast of Mexico: California spiny lobster (*Panulirus interruptus*, west coast of California and the Baja California Peninsula); blue spiny lobster (*P. inflatus*, southern Baja California Peninsula to the State of Oaxaca, Mexico); and green spiny lobster (*P. gracilis*, a tropical species from southern Baja California Peninsula to Peru) (Hendrickx, 1995). Taxonomic identification of adult lobsters is easily done by morphological features (Hendrickx, 1995); however, alternative techniques are required for identification of larvae when morphological features are unable to provide the means of identifying early life stages of spiny lobster species (Johnson, 1971; Muñoz-García et al., 2004). Furthermore, discrimination between larvae of *P. inflatus* and *P. gracilis* could be especially difficult because of their overlapping distribution.

The species-specific identification of larvae of other *Panulirus* species has also been difficult. Chow et al. (2006b) found intraspecific and intra-individual variation in appendage

structures (the subexopodal spines) in the phyllosoma of *P. ornatus* (ornate rock lobster) and *P. versicolor* (painted spiny lobster). Therefore, subexopodal spine arrangements may not be a useful diagnostic for distinguishing between these two species. Because of these potential problems, several authors have suggested the use of molecular markers to identify spiny lobster larvae (Silberman and Walsh, 1992; Chow et al., 2006a, 2006b; Konishi et al., 2006).

In this study, the nucleotide variations of the mitochondrial DNA (mtDNA) in adult lobsters were investigated to obtain genetic markers useful in identifying *P. interruptus*, *P. inflatus*, and *P. gracilis* through either the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis or species-specific primers that amplify different size fragments in a multiplex reaction. These techniques were used to identify phyllosoma larvae collected outside the Gulf of California.

Materials and methods

PCR-RFLP analysis

DNA from adult specimens of the three species was taken from the following sites on the west coast of Mexico: *P. interruptus* from Baja California ($n=2$) and Baja California Sur ($n=2$); *P. inflatus* from Baja California Sur ($n=3$), Sinaloa ($n=4$), Nayarit ($n=2$), and Jalisco ($n=1$); and *P. gracilis* from Baja California Sur ($n=2$), Sinaloa ($n=3$), and Nayarit ($n=1$) (see Table 1).

A fragment of the 16S rRNA gene was amplified with primers 16Sar-L (5'-CGCCTGTTTATCAAAAACAT) and 16Sbr-H (5'-CCGGTCTGAACT-CAGATCACGT) (Palumbi, 1996).

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Table 1

Sampling sites and dates for each lobster species along the Pacific coast of California and Mexico. The number of samples in the Seq1-16S column refers to the sequences used to find diagnostic restriction sites to discriminate lobster species; RFLP and Multiplex columns are the number of larvae analyzed by the RFLP and multiplex analysis, respectively; Seq2-16S column are the sequences used to evaluate consistency of restriction patterns.

Sampling sites	Location	Sampling date	Number of samples			
			Seq1-16S	RFLP 16S	Multiplex 12S-CR	Seq2-16S
<i>Panulirus interruptus</i> (California spiny lobster)						
Catalina Island, CA, USA	33°26', 118°29'	Mar 2003			1	
Ensenada, B.C., Mexico	31°50', 116°38'	Aug 1999	1	3	1	
Isla Guadalupe, B.C., Mexico	29°00', 118°10'	Dec 2002	1	3	1	
Punta Eugenia, B.C.S., Mexico	27°49', 115°06'	Jun 1999		2	1	
Punta Abreojos, B.C.S., Mexico	24°41', 113°34'	Jun 1999		2	1	
San Juanico, B.C.S., Mexico	24°14', 112°27'	Oct 1999		2	1	
Bahía Magdalena, B.C.S., Mexico	24°46', 112°06'	Dec 1999	2	2	1	
<i>Panulirus inflatus</i> (blue spiny lobster)						
Bahía Magdalena, B.C.S., Mexico	24°46', 112°06'	Nov 2001	3	3	1	21
Mazatlán, Sin., Mexico	23°13', 106°26'	Aug 2002	4	3	1	14
Las Peñitas-Sayulita, Nay., Mexico	21°00', 105°23'	Aug 2002	2	3	1	24
Barra de Navidad, Jal., Mexico	19°12', 104°42'	Jan 2005	1	3	1	15
Zihuatanejo, Gue., Mexico	17°37', 101°33'	May 2005		2	1	29
Puerto Angel, Oax., Mexico	15°39', 96°29'	Nov 2002			1	23
<i>Panulirus gracilis</i> (green spiny lobster)						
Bahía Magdalena, B.C.S., Mexico	24°46', 112°06'	Nov 2001	2			
Mazatlán, Sin., Mexico	23°13', 106°26'	Aug 2002	3	4	2	21
Las Peñitas-Sayulita, Nay., Mexico	21°00', 105°23'	Aug 2002	1	5	1	23
Punta Maldonado, Gue., Mexico	16°20', 98°34'	May 2005		5	2	25
Total			20	42	18	195

PCR was performed in a total volume of 25 μ L (Invitrogen 1 \times PCR buffer, 0.2 mM dNTP mix, 0.48 μ M of each primer, 4.0 mM MgCl₂, 1.25 U *Taq* DNA polymerase), with an iCycler thermal cycler (Bio-Rad Laboratories, Hercules, CA). The PCR program consisted of a denaturation step at 94°C for 2 min, followed by 30 cycles of 1 min at 94°C, 1 min at 60°C, and 2 min at 72°C, followed by a final extension step of 4 min at 72°C. PCR products were confirmed using electrophoresis on 1% agarose gel, along with a molecular weight marker to estimate the fragment size. The gel was stained with SybrGold (Molecular Probes, Eugene, OR). All amplified products were sequenced with primers 16Sar-L and 16Sbr-H (Macrogen, Korea) and deposited in GenBank (accession numbers EF546597 through EF546616).

The 16S rRNA gene sequences were aligned and edited with the program Sequencher, vers. 4.5. (Gene Codes Corporation, Ann Harbor, MI). A neighbor-joining phylogram of haplotypes, based on the Kimura-2 parameter model, was constructed in Molecular Evolutionary Genetics Analysis (MEGA) software vers. 3.0 (Kumar et al., 2004). Three sequences (one for each species) were used for the identification of diagnostic restriction

enzyme recognition sites in ChromasPro software, vers. 1.33 (Technelysium Pty Ltd, Tewantin, Queensland, Australia). Even though several restriction enzymes revealed various cut sites in the sequences, we selected only those that were easily seen on agarose gel. To check for consistency of the restriction enzyme cutting pattern in each species, the analysis was done with 17 additional sequences.

The RFLP pattern of the selected enzymes of another 42 adult lobster specimens of the three species was tested for specimens collected at different locations (Table 1). Each digestion reaction occurred in a final 7 μ L volume with 0.7 unit/ μ L of the selected enzyme, 3.5 μ L PCR product, and according to the manufacturer's instructions (New England Biolabs, Ipswich, MA). Reaction products for each enzyme were incubated for eight hours at the optimal temperature suggested by the manufacturer. For electrophoresis, restricted products were run on 2% agarose gels and stained with SybrGold. A molecular weight marker was added to the agarose gel to estimate fragment size. To check consistency of intra- and interspecific nucleotide variation in addition to the 20 sequences, 195 sequences (a total

of 215) from different geographical sites of *P. inflatus* ($n=126$) and *P. gracilis* ($n=69$) were analyzed. This extension was important because the larvae of these species are found in the same area and there is a higher possibility that misidentification will occur when using only morphological criteria (Table 1).

Panulirus penicillatus (pronghorn spiny lobster) is found along the Pacific coast of Mexico and even though it is found in small numbers, it could be confused with the larvae of the other species. The restriction pattern of a 16S rRNA gene sequence reported in the GenBank (accession number AF337974) was compared to those of the other three lobster species.

Multiplex PCR analysis

Species-specific primers for *P. interruptus* have been described in a previous study (García-Rodríguez and Perez-Enriquez, 2006; GenBank accession number EF565146). Based on this sequence, a reverse primer (5'-TGGTGTGATCCCGTTACTTG) was designed to amplify a ~1250 base-pair (bp) mtDNA fragment containing the 12S rRNA gene and the control region in *P. inflatus* and *P. gracilis* by means of Weider et al.'s (1996) forward primer (srRNA: 5'-CAGGGTATCTAATCCTGGTT). PCR thermal cycling consisted of an initial denaturation of 2 min at 94°C, 35 cycles of 1 min at 94°C, 1 min at 58°C (for *P. inflatus*, but 52°C for *P. gracilis*), 2 min at 72°C, and a final cycle of 4 min at 72°C. PCR products from adult specimens of each species were sequenced by using the srRNA primer. The Primer3 program (Rozen and Skaletsky, 2000) was used to design specific primers for each species; these primers would have similar melting temperatures but products of different sizes (Fig. 1). Sequences obtained with specific primers were deposited in GenBank (accession numbers EF565144 and EF565145).

The specificity and reliability of the multiplex PCR reaction with 18 previously identified adult lobsters collected in different regions were tested. For *P. interruptus* ($n=7$), specimens were obtained from California, Isla Guadalupe, Baja California, and Baja California Sur; for *P. inflatus* ($n=6$), specimens were collected from Baja California Sur, Sinaloa, Nayarit, Jalisco, Guerrero, and Oaxaca; for *P. gracilis*, specimens ($n=5$) came from Sinaloa, Nayarit, and Guerrero (Table 1).

Multiplex PCR reactions were carried out in a total volume of 12.5 μ L, mixing 0.48 μ M of each primer (one common primer: srRNA, and the three species-specific primers: LanCR-R (5'-AAAAATTCAGGCTAATGGA), PinRC1-b (5'-GATGGCCCATTACCGAATA), and PgraRC1-b (5'-TTGTGAAACGTCTGTTTACATTATTT)), Invitrogen 1 \times PCR buffer, 0.2 mM dNTP mix, 4.0 mM MgCl₂, and 0.625 U Taq DNA polymerase. PCR thermal cycling consisted of an initial denaturation of 2 min at 94°C, followed by 30 cycles of 1 min at 94°C, 1 min at 59°C, 2 min at 72°C, and a final cycle of 4 min at 72°C. The amplified products from each species were distinguished by electrophoresis on 1.0 % agarose gels.

Application of molecular markers

Phyllosoma larvae were collected during an oceanographic cruise outside the Gulf of California in November 2004. Plankton sampling consisted of horizontal surface tows of a neuston collection net at 3.5 knots (6.4 km/h) for 5 min (see González-Armas et al., 1999). Sampling gear consisted of a rectangular plankton net of Nylax with 505- μ m mesh; it had a 30 \times 50 cm mouth area and the net was 3 m long. Phyllosoma larvae were sorted by hand after each tow and fixed with 70% ethanol.

A fragment of the pereopods, antenna, or eyes was obtained from each lobster larva for DNA isolation by lysing the tissue in 15 μ L lysis buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 0.5% Tween-20), and 1.87 μ L (4 μ g/ μ L) proteinase K and incubated overnight at 55°C. Each reaction was then maintained at 95°C for approximately 10 min and stored at 4°C until analysis. Steps of PCR-RFLP and PCR multiplex for larval lobsters were the same as those carried out for adult specimens.

Before genetic analysis, 46 lobster larvae collected from the Pacific Ocean were categorized into four possible groups based on morphological criteria (Johnson and Knight, 1966; Johnson, 1971; Báez, 1983): group 1) *Panulirus inflatus*-like ($n=17$); group 2) *Panulirus gracilis*-like ($n=8$); group 3) *Panulirus inflatus-gracilis*-like ($n=12$); and group 4) *Panulirus*-like ($n=9$).

Results

PCR-RFLPs

The 16S rRNA gene fragment was correctly amplified in the three lobster species. The size of the 16S rRNA gene fragment amplified in the three spiny lobster species was estimated at 563 bp and did not differ in size among species. There was wide inter- and intraspecific variation in nucleotide sequences among the lobster species, indicating the potential for species discrimination. A haplotype tree showed the aggregation of haplotypes according to each species (Fig. 2).

From the analysis of 20 sequences, two restriction enzymes (*Bsm*AI, GTCTCN' and *Hinf*I, G'ANTC) were selected that allowed discrimination among the three species (Fig. 3). The restriction products of the *Bsm*AI digests were two fragments (~401 and ~162 bp) in both *P. interruptus* and *P. inflatus* (named "haplotype A") and three fragments (~401, ~115, and ~47 bp) in *P. gracilis* (named "haplotype B"). Restriction digests that used *Hinf*I produced two fragments (~440 and ~123 bp) for *P. interruptus* and most *P. gracilis* specimens (named "haplotype A"), but did not cut *P. inflatus* (named "haplotype B"). One *P. gracilis* sequence appeared to be haplotype B due to the absence of the *Hinf*I site. The composite haplotypes were constructed by the combination of the haplotype names of each enzyme, resulting in AA for *P. interruptus*, AB for *P. inflatus*, and either BA (98.7%) or BB (1.3%) for *P. grac-*

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1
P. ine-ENS1 TACAGTTACTTGATTACTAACACTTGCCAATATACAAATTCACCTCTATTGTTTCCAGTACTGAATATAAATG-AACTTAA--TTGTCTAACTTTATAA
P. inf-JAL1 -----T-----A-----A-----A.A.TA.AC..AT..C.TT..TT....CGGT.CA.T...C..TAC.
P. gra-GUE3 ---CT.GCGC.C.A..TT...-T-----A-----T.A....A.A.TG..C..AT.....T..TC....CGTT.CA.T...C..TAT.

103
P. ine-ENS1 CTTACTCTTTTTCAGTGGCCTTCAAGTCTAACCGGGATGTGGCACAAGTTTAACTGACCTAAATGTTTTTACTTAATCCAATCTTTATTGATT-AT
P. inf-JAL1 ...T..ACC.....A..G.....C.....G.G.A.GC..G.....CA...ATC...T..
P. gra-GUE3 ...TC.ACC.....A..A..G.....C.....A.GC..G.....CAT..AT.....T..

205
P. ine-ENS1 TAAATTTTGTACTGCTCATGTAATATAGCGTTAGCTTATTATTCGA-----CTCACTTAAATATTGAATTAAAGTGCTATATTTTAAATGACT
P. inf-JAL1 .....A.....A.C.C....AAT.GTA..C.CAC..CC.A...CGTCGCACC.C.CTC..CG...T..CG..CAGTG..G..AA...-CAA...
P. gra-GUE3 .....A.....A.T.TC...AAT..TA..C.TTAC..CC...TT.--CACACC.ATTT.CCCG...CT..CG..C.GTG.C...AA...-CAA...

307
P. ine-ENS1 AATATTTGCATGTACTATTCAAACCAATAGCGAAAAGAAATAGCAAGAATCAAATATAGGACCTACCACTTATATTTTACTTTAATATTGATTTT
P. inf-JAL1 ..A.....T.T....G....C..AT-.G.T.....TA.ATG..CAA...-G.CC.C.TCGACCC...-AAA
P. gra-GUE3 ..A.....T.T....G....AT-.G.T.....CT.TACA..CAAGG.-.A.AC.A.T.AATCA...T...AA

409
P. ine-ENS1 AATTTTA-ATAGTAGTTTTTAAAGAACTAACACTAATAGATGACCTTTTATACCTATTA--CCGGCTAAAAGTTTAGTAAAAATACAATCTACTGATATTAT
P. inf-JAL1 .T.A.A.C....AC..A....T.TTTT..T..A.ATGTT--A.C...A.AA.ACT.ATT.TTC.ACGCCCC..GTTAC...A.GTT.AGCGACTA
P. gra-GUE3 .CAAATGT.T...T.AA.G....TATTTTACT..A.AT.AT..AA..TTTA.AAGATTTAAT..C-A..ATCC.GCTTAC...A.GCT.A.CG..GA

511
P. ine-ENS1 TCCCACAGACTTAGAGGACCAAAAATGGAA---GGAAATTTAAAATGCGCCC-ATTAAC TAGTATTTCGGTTAACAAGTTATTTACCATTATATATACAT
P. inf-JAL1 CT.G.T.TCG.A.C.-.AA.TCCG..TA..GCCCTA.T.ACC..-CAATT.AAG.C...CGAC...CAA.TTTTTC.T...-T.T.....
P. gra-GUE3 C..G.T.CT..CT...AA.TTC..ATT..-CCAA.T.....TCATT.TAAG.C.G.CCTGT...CAACC.ATTAC.T...-TTTG.....

613
P. ine-ENS1 TTAAAGAAAATGTAATTAGACCGTTTACAATAAGTACAGGGCTT--ATTTTTCACCAATTAATAAATAAATAAATAA-AAGGCTTATACTAATTTAAACA
P. inf-JAL1 ...TT.T.-.....G....TA..A...TCAAG...A.A...AA.TAC.....A..T....CTG.A.TG...CT..TGA.C.TTG
P. gra-GUE3 ..AAATAAA-TGTAACAGAC-GTTTCACAA..TA..A..A.TCAAG...A.A...AA.TAC.....T...CC-.A.TA...CT..TG..T..TT

715
P. ine-ENS1 CCATATCTAAAGAGTAACAATTATAGCATGTATTAAACAAGTACGATATGGCTTCCTTCTAAAAAAGGTTAAATTTGGAGATAGATTAGAGCCGATTTTGTA
P. inf-JAL1 TT...AGCTCC...-.....A---G..C.GTGAGTTCGGTAATGGG-CCATCG-.C..TTC.C..G.....
P. gra-GUE3 .T...AA...C...TG...A---G.ACTGTTT..GT..GC.AT.GG-.C.C.C...TTC.C..G...T...GAC..T-----

817
P. ine-ENS1 CTTTACCCCGAGGAAGTACTGTAAATGCGGCTCTAATCTATCCACATATACTAATGAAGCCTTTATAAACCATTTCAGCGAATATAACTTATTATTAGAT
P. inf-JAL1 -----
P. gra-GUE3 -----

919
P. ine-ENS1 TATACACCTTATTGTATGTTTAAATAAACTTAAATGTATATATATCTAGAGATATCCATTAGCCCTGAATTTTGTAGCCCTCCCCCCTTCTCATCCTCTA
P. inf-JAL1 -----
P. gra-GUE3 -----

1021
P. ine-ENS1 TCTGAGTTTATTTCTTAAATTAATACAATTTTATATTTGGCTGGCGTGGTTAGTTTATATTTAATTAATATTTTGGCCATTAAACATGATGCCTGATAAAAG
P. inf-JAL1 -----
P. gra-GUE3 -----

1123
P. ine-ENS1 GTTTGTGTTTGTAGACAAAGTAATGCAGATAAAAAGTCTCATGTTTACAAGTAAACGGGATCACACCAATTCCTAGAAATCAAAAATCTTATGCTC
P. inf-JAL1 -----
P. gra-GUE3 -----

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Figure 1

Alignment of control region sequences of California spiny lobster (*Panulirus interruptus* [*P. ine*]), blue spiny lobster (*P. inflatus* [*P. inf*]), and green spiny lobster (*P. gracilis* [*P. gra*]) used to find specific primers from one specimen of each lobster species. Underlined regions indicate species-specific primers. Label after the species name indicates specimen's voucher code. Numbers 1, 103, 205, ..., 1123, indicate positions in the sequence of the first nucleotide in the row.

ilis (Table 2). Thus the composite haplotypes are able to delineate among these species. All electrophoretic patterns of PCR-RFLP products of adult specimens were congruent with those obtained from sequences analysis. Finally, the sequence of *P. penicillatus* had restriction sites that would produce different patterns from the other species (Fig. 3).

Multiplex PCR

A high annealing temperature (59°C) was adequate for successful multiplex PCR reactions containing the DNA of the three species. There were no additional fragments to those expected that could prevent identification of lobster species in all tested adult specimens. The size of

Table 2

Mitochondrial DNA (mtDNA) 12S rRNA-control region (563 base pairs [bp]) fragment sizes resulting from the *Bsm*AI and *Hinf*I-recognizing sites during nucleotide sequence analyses of three *Panulirus* species: California spiny lobster (*Panulirus interruptus*), blue spiny lobster (*P. inflatus*), and green spiny lobster (*P. gracilis*). Hap = haplotype nomenclature (see text for explanation); % = percentage of haplotypes in a sample of 215 sequences. Composite haplotypes were obtained by combining the haplotype of each enzyme.

Species	Enzyme	Fragment size (bp)	Hap	%	Composite haplotype
<i>P. interruptus</i>	<i>Bsm</i> AI	162,401	A	100.0	AA
	<i>Hinf</i> I	440,123	A	100.0	
<i>P. inflatus</i>	<i>Bsm</i> AI	162,401	A	100.0	AB
	<i>Hinf</i> I	563	B	100.0	
<i>P. gracilis</i>	<i>Bsm</i> AI	115,47,401	B	100.0	BA / BB
	<i>Hinf</i> I	440,123	A	98.7	
		563	B	1.3	

the amplified product delineated all three species in a 1% agarose gel. The fragment size of *P. interruptus* (with the use of LanCR-R primer) was ~1000 bp, the fragment size of *P. inflatus* (with the use of PinRC1-b) was ~800 bp, and the fragment size of *P. gracilis* (with PgraRC1-b) was ~700 bp (Fig. 4). Even though two *P. interruptus* specimens produced unspecific amplifications, the fragments stained weakly compared to the 1000-bp fragment and did not interfere with identification. Because the specimens analyzed by multiplex PCR were collected at

different sites, these data show that there is no apparent intraspecific variation, which indicates that the multiplex primer set provides a method that can be used to identify the three lobster species.

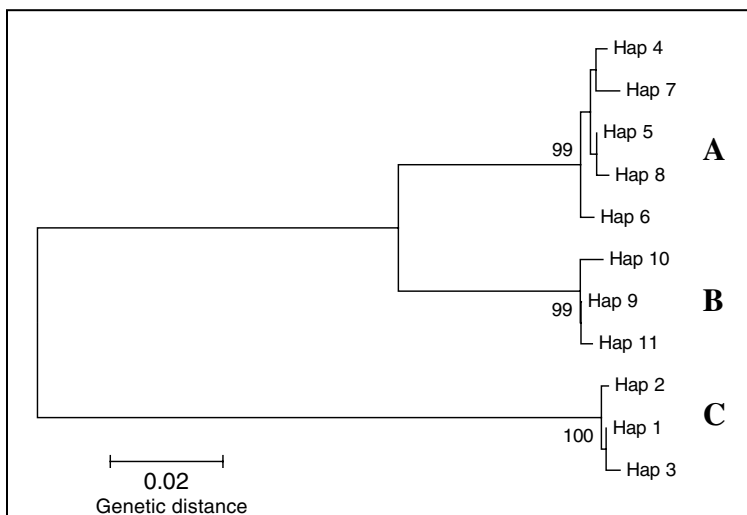
Larval identification

Species identification based on PCR-RFLP did not support the identification based on morphological criteria. The fragment patterns produced by digestion with *Bsm*AI and *Hinf*I provided a means for identifying *P. inflatus* and *P. gracilis*. Two specimens in each of groups 1 and 2 were identified as *P. gracilis*. The 42 remaining larvae belonging to groups 1, 2, 3, and 4 were identified as *P. inflatus*; none of the specimens were identified as *P. interruptus*. Multiplex PCR analysis confirmed the results.

Discussion

Several methods with molecular markers have been carried out for identification at the species level. Even though PCR-RFLP and multiplex-PCR are widely used as tools in distinguishing species of different taxonomic groups (Moore et al., 2003; Chow et al., 2006a), efforts have to be made to obtain the proper amplification primers when universal primers do not give consistent results in the studied species.

According to our analysis, identification of spiny lobster species can be successfully done by combining different strategies. First, although insufficient for recognizing spiny lobsters larvae, morphological criteria should be used. Then, one or both of the genetic techniques can be applied to definitively support the morphological results. The simultaneous use of the PCR-RFLP and multiplex PCR

**Figure 2**

Neighbor-joining phylogenetic tree based on the mitochondrial 16S rRNA gene haplotypes (Hap 1–11) from a total of 20 adult lobsters of blue spiny lobster (*Panulirus inflatus*), green spiny lobster (*P. gracilis*), and California spiny lobster (*P. interruptus*). Haplotype groups identified as A, B, and C represent blue, green, and California spiny lobster, respectively. Tree reconstruction was based on Kimura's two-parameter distance (K2P) with 1000 replications. Numbers on the nodes are bootstrap values. The branch length is measured as the number of nucleotide substitutions.

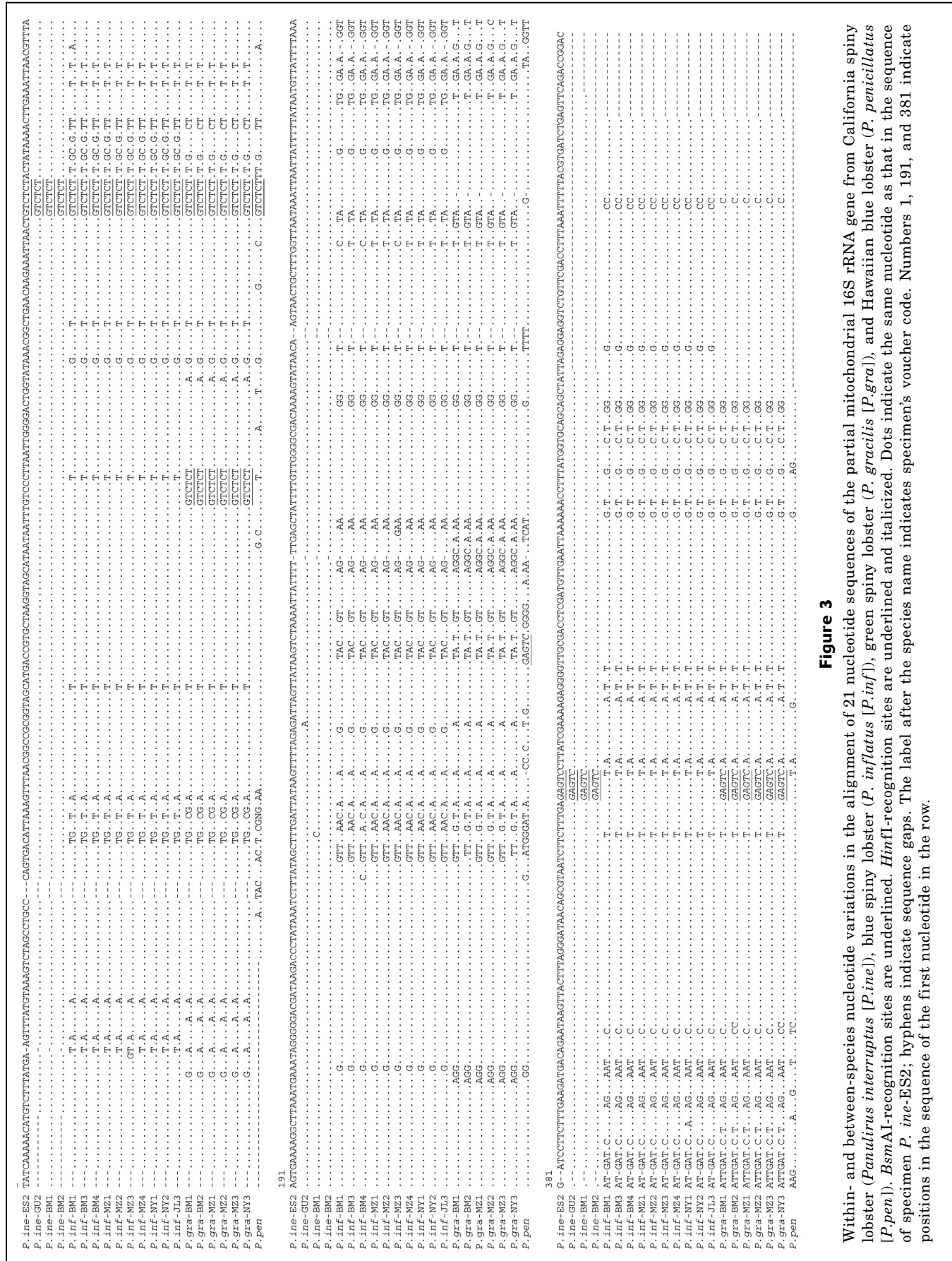
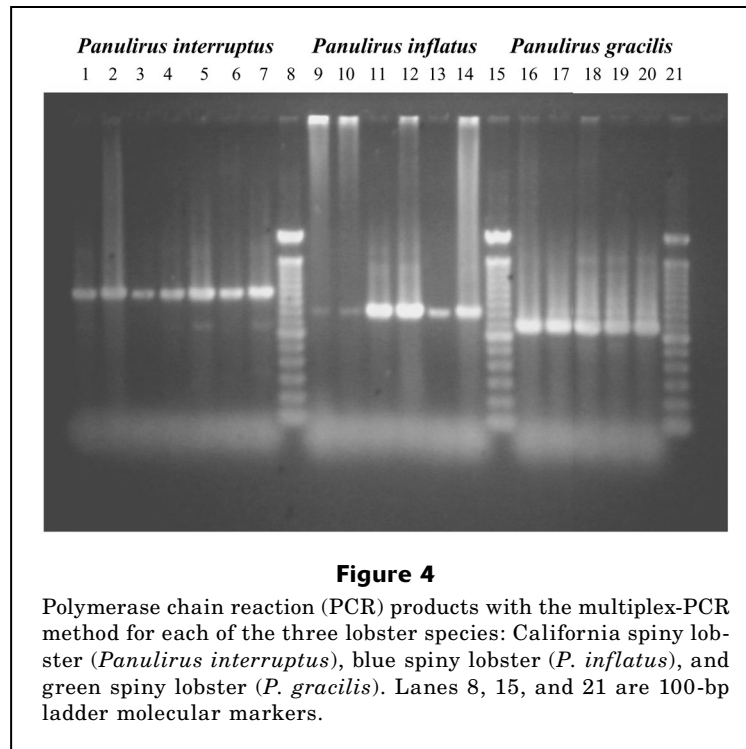


Figure 3

Within- and between-species nucleotide variations in the alignment of 21 nucleotide sequences of the partial mitochondrial 16S rRNA gene from California spiny lobster (*Panulirus interruptus* [*P.ine*]), blue spiny lobster (*P. inflatus* [*P.inf*]), green spiny lobster (*P. gracilis* [*P.gra*]), and Hawaiian blue lobster (*P. penicillatus* [*P.pen*]). *Bsm*AI-recognition sites are underlined. *Hinf*I-recognition sites are italicized. Dots indicate the same nucleotide as that in the sequence of specimen *P. ine-ES2*; hyphens indicate sequence gaps. The label after the species name indicates specimen's voucher code. Numbers 1, 191, and 381 indicate positions in the sequence of the first nucleotide in the row.



techniques is recommended because there are instances for which unsuccessful amplifications are obtained by one of the methods. This is a consequence of mutations in the annealing sites, rather than a failure of PCR techniques (Ray et al., 2002).

The 20 sequences that we evaluated for differences in restriction patterns revealed species-specific restriction sites for *Bsm*AI and *Hinf*I. Amplified products of previously identified adult lobsters were concordant with the restriction patterns found in these sequences. The PCR-RFLP technique is successful for separating *Panulirus* larvae because 16S rRNA is relatively easy to amplify at intraspecific conservative sites and interspecific variable sites.

The PCR-RFLP has also been applied to the cytochrome oxidase I (COI) gene to identify 10 spiny lobster species of the genus *Panulirus* found in the northwestern Pacific Ocean (Chow et al., 2006a). In general, Chow et al. (2006a) found more than two haplotypes per species; however, no composite haplotype was shared by these species. Although Chow et al. (2006a) suggested increasing efforts in searching for intraspecific variation on larger samples, the probability of misidentification is very low because substantial divergence has been observed among species of the genus *Panulirus* (Ptacek et al., 2001). Our results support the finding of high divergence among the three lobster species in Mexico on the basis of nucleotide intraspecific variation (Fig. 2).

Multiplex-PCR fragments are clearly capable of discriminating among the three lobster species. This method is fast, simple, and relatively inexpensive because

species identification can be performed by using just PCR amplifications with no digestion and with small reaction volumes. The multiplex-PCR method used in this study is successful because it is based on species-specific primers and a sufficiently high annealing temperature (T_m) of 59°C is used that avoids the amplification of unspecific PCR products. Also, a high T_m reduces the possibility of amplifying homologous sequences from other species.

Identification of lobster larvae is consistent with the restriction patterns found in adult lobsters and identifications carried out by PCR-RFLP can be correctly confirmed by using multiplex PCR. Molecular analyses showed that the previous larval classification (based on morphological characters) was probably incorrect because very small or injured specimens were used. Alternatively, misidentification in lobster larvae could also be a consequence of morphological criteria that are not diagnostic characters for discriminating between lobster larvae, as it has been reported in other *Panulirus* species, such as the Japanese spiny lobster (*P. japonicus*) and species from the Atlantic Ocean (i.e., the Caribbean spiny lobster, *P. argus*) (Silberman and Walsh, 1992; Chow et al., 2006a, 2006b). Morphological criteria currently used for the identification of phyllosoma larvae of *P. interruptus*, *P. inflatus*, and *P. gracilis* are insufficient as well. Further descriptions of larvae specimens should be conducted to search for consistent morphological differences between species.

Other possible applications of PCR-RFLPs and multiplex PCR are used in fishery forensics, when lobster

products must be identified. This is especially the case when lobster products of unknown origin have to be analyzed if there is suspicion that the lobster was obtained by illegal fishing, as in the case of important protected species, such as sea turtles (Moore et al., 2003).

Conclusions

We used the nucleotide variation of two mtDNA fragments from adult spiny lobster samples to find molecular marker applications for species discrimination. The RFLP and multiplex-PCR protocols developed in this study allow for correct discrimination of three commercial lobster species inhabiting the Pacific coast of Mexico. Application of both types of molecular markers in the identification of lobster larvae showed concordance and the potential to discriminate between phyllosoma species during early stages. Molecular identification of larvae was inaccurate with our previous assignment based on morphological criteria. Thus, the use of morphological characteristics in phyllosoma larvae can be misleading for identification to the species level because anatomical parts can easily be damaged during collection. This new information on genetic identification of species at the larval stage is of wide interest because studies focused on taxonomic and ecological revisions require accurate species identification.

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